

ONCO/Reveal™ Myeloid Panel Reference Guide

Library Preparation User Guide

FOR RESEARCH USE ONLY

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INTRODUCTION

The ONCO/Reveal™ Myeloid Panel utilizes our proprietary SLIMamp® (stem-loop inhibition mediated amplification) technology, allowing researchers to amplify regions of interest in a simple, single-tube, multiplex reaction. Subsequent libraries are designed for sequencing on the Illumina platform using a paired-end read length of 150 (2x150) or 275 (2x275). The ONCO/Reveal Myeloid Panel contains numerous gene regions of interest for researchers looking to explore the genetic sequences myeloid cancer samples.

The workflow of the ONCO/Reveal Myeloid 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 Pillar ONCO/Reveal Myeloid Panel Work?

A pair of DNA oligos designed for each region of interest, or hot spot, are used 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).

Limitations

The ONCO/Reveal Myeloid Panel can detect internal tandem duplications (ITD) in the FLT3 gene. An ITD up to 126 bases can be detected using paired end sequencing at read lengths of 175 bp (2x175).

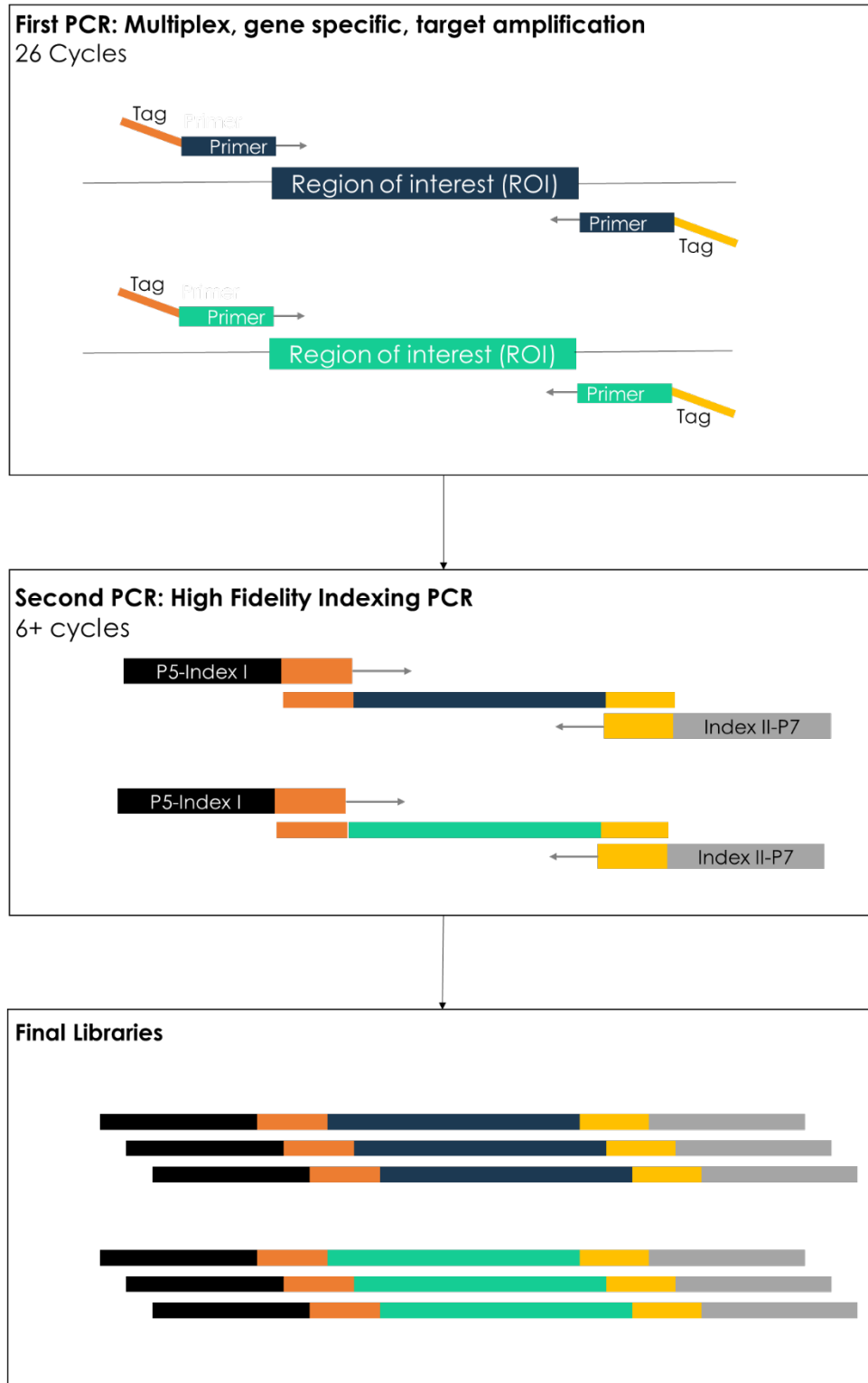


Figure 1. Overview of the ONCO/Reveal Myeloid Panel library preparation.

REVISION HISTORY

2019-04: Creation

2019-05: Minor grammatical edits

2019-06: Minor grammatical edits

2019-07: Updated Indexing PCR reaction

2020-05: Limitations section added in version 0.3.5

2020-09: Edits to improve consistency and clarity in version 0.3.6

GETTING STARTED

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

Components of the ONCO/Reveal Myeloid Panel

Reagent	Use	Area Use	Storage
<i>Gene-Specific PCR Master Mix (2x)</i>	Gene-specific PCR	Pre-PCR	-15° to -25°C
<i>Myeloid oligo pool</i>	Gene-specific PCR	Pre-PCR	-15° to -25°C
<i>GC Rescue G</i>	Gene-specific PCR	Pre-PCR	-15° to -25°C
<i>Exonuclease I</i>	Gene-specific PCR	Post-PCR	-15° to -25°C
<i>GC Rescue D</i>	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Indexing PCR Master Mix (2x)</i>	Indexing PCR	Pre-PCR	-15° to -25°C

ONCO/Reveal Myeloid Panel Indexing Kits

Reagent and Part Number	Use	Area Use	Storage
<i>Pillar Custom Indexing Primers Kit A, indices PI501-8, PI701-4 (32 combinations - 96 reactions) PN: IDX-PI-1001-96</i>	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Pillar Custom Indexing Primers Kit B, indices PI501-8, PI705-8 (32 combinations - 96 reactions) PN: IDX-PI-1002-96</i>	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Pillar Custom Indexing Primers Kit C, indices PI501-8, PI709-12 (32 combinations - 96 reactions) PN: IDX-PI-1003-96</i>	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Pillar Custom Indexing Primers Kit D, indices PI501-8, PI701-12 (96 combinations - 192 reactions) PN: IDX-PI-1004-192</i>	Indexing PCR	Pre-PCR	-15° to -25°C
<i>Pillar Custom Indexing Primers Kit E, indices PI501-8, PI701-12 (96 combinations - 384 reactions) PN: IDX-PI-1005-384</i>	Indexing PCR	Pre-PCR	-15° to -25°C

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

All reagents in the kit should be used in designated pre-PCR or post-PCR areas to prevent amplicon contamination. Each area designated for pre-PCR and post-PCR should have dedicated equipment, supplies, and reagents to prevent contamination.

User-supplied Reagents

Reagent	Area use	Supplier
10 N NaOH or 1 N NaOH	Post-PCR	General lab supplier
Agencourt AMPure XP Beads	Post-PCR	Beckman Coulter, #A63881/ #A63880
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 kit v2 (300 cycles)	Illumina, #MS-102-2002
MiSeq reagent kit v3 (600 cycles)	Illumina, #MS-102-3003
NextSeq 500/550 Mid Output v2 kit (300 cycles)	Illumina, #FC-404-2003

Consumables

Item	Area Use	Supplier
1.5 mL microcentrifuge tubes	Pre- and post-PCR	General lab supplier
96-well PCR plates, 0.2 mL	Pre- and post-PCR	Axygen, #6551 or equivalent
Microplate sealing film	Pre- and post-PCR	Axygen, #PCR-TS or equivalent
Conical tubes, 15 mL	Pre- and post-PCR	General lab supplier
Conical tubes, 50 mL	Post-PCR	General lab supplier
Low retention, aerosol filter pipette tips	Pre- and post-PCR	General lab supplier

<i>Solution basin (trough or reservoir)</i>	Pre- and post-PCR	Fisher, #13-681-506 or equivalent
<i>Qubit Assay tubes</i>	Post-PCR	Invitrogen, #Q32856

Equipment Requirements

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

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

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

BEST PRACTICES

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

- **Work areas:** To reduce the risk of contamination from PCR amplicons, supplies should not be moved from one area to another. Separate storage areas (refrigerator, freezer) should also be designated for pre- and post-PCR products.
- **Lab cleanliness:** To further reduce the possibility of contamination, clean work areas between experiments with laboratory cleaning solution (70% alcohol or freshly-made 10% hypochlorite solution). A periodic cleaning of the floor is also recommended.
- **Floor:** Items that have fallen to the floor are assumed to be contaminated and should be discarded. Gloves should also be changed after handling a contaminated item. If a sample tube or non-consumable item has fallen and remained capped, thoroughly clean the outside with a laboratory cleaning solution before use (70% alcohol or freshly-made 10% hypochlorite solution).
- **Aliquot reagents:** Aliquot frozen reagents into smaller volumes to prevent freeze/thaw cycles. For reagents stored at higher temperatures, aliquot from the stock and work from the aliquots to reduce the risk of stock contamination. In the case of contamination, aliquots can help to determine the source of contamination more quickly and easily.
- **Multichannel pipettes:** Use multichannel pipettes for consistency and efficiency among numerous samples.
- **Pipette tips:** Change tips between each sample to prevent cross-contamination. Discard any tips that may have become contaminated due to contact with gloves, lab bench, tube exteriors, etc.
- **Open containers and lids:** To prevent possible contamination from the air, keep tubes closed when not directly in use, avoid reaching over open containers, and cover plates with seals or lint-free laboratory wipes.

ONCO/Reveal Myeloid Panel Workflow

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

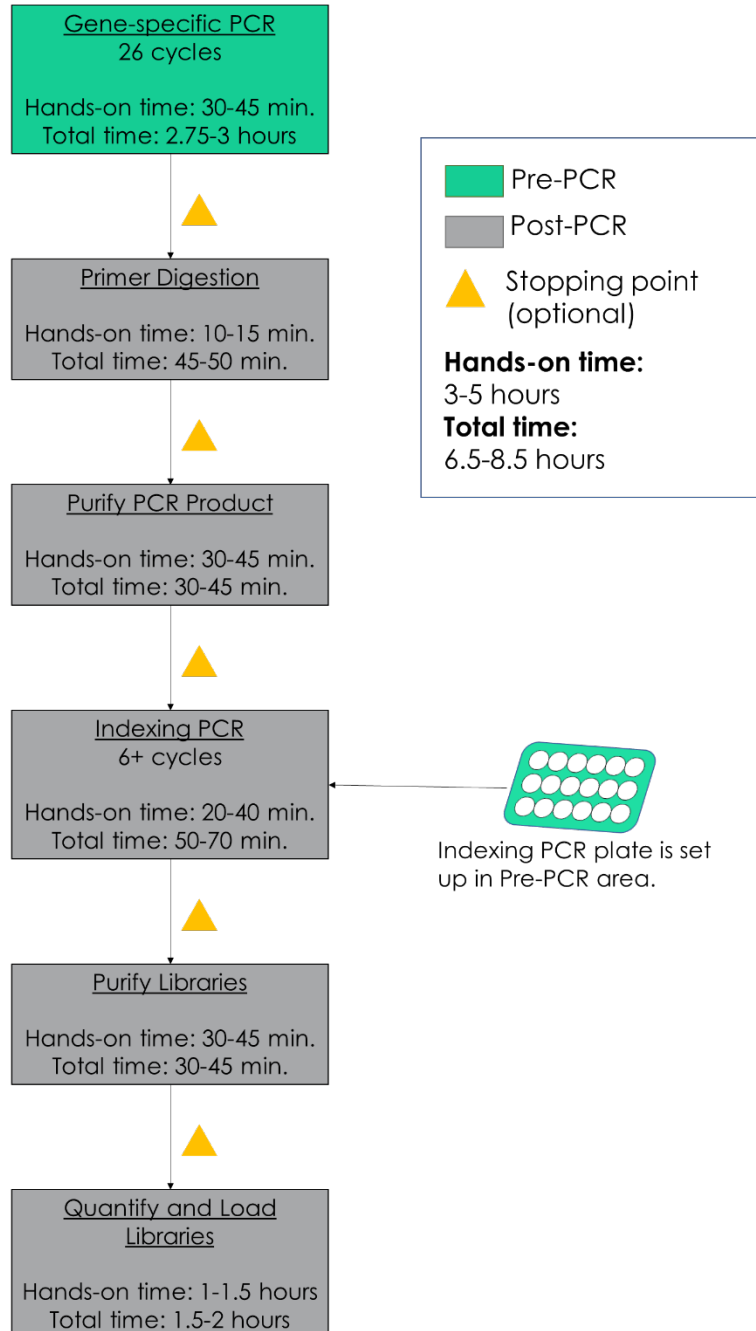


Figure 2. The ONCO/Reveal Myeloid Panel workflow can be completed within a day but contains multiple stopping points for users with time constraints.

LIBRARY PREPARATION PROTOCOL

Hands-on time: 3-5 hours

Total time: 6.5-8.5 hours

Gene-specific PCR: Amplify Genomic DNA Targets

Hands-on time: 30-40 minutes

Total time: 2.75-3 hours

The following steps are performed in a pre-PCR area. For this portion of the protocol, have an ice bucket prepared. Keep the Gene-specific PCR MasterMix (GS PCR MMX) and custom 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 below.

Reagent	Volume (µL)
Gene-specific PCR Master Mix	12.50
Myeloid oligo pool	7.00
GC Rescue G	1.25
Sub-Total	20.75

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

2. **Transfer:** Transfer 20.75 µL of master mix to each sample well in a PCR plate, strip tube, or PCR tube.
3. **Dilute input DNA:** Dilute DNA in nuclease-free water to a final volume of 4.25 µL of diluted genomic DNA. The recommended DNA input range is 20-60 ng*. Add 4.25 µL of diluted DNA to each sample well contain PCR master mix. Add 4.25 µL of nuclease-free water to the no-template control well.

*The DNA concentration can be determined by the Qubit dsDNA BR Assay Kit (Life Technologies, Cat. No. Q32850 or Q32853; quantitation range 2-1,000 ng) or the Qubit dsDNA HS Assay Kit (Life Technologies, Cat. No. Q32851 or Q32854; quantitation range 0.2-100 ng). Recommended input range is 20-60 ng of genomic DNA.

Reagent	Volume (µL)
PCR master mix	20.75
Diluted DNA (or water)	4.25
Total	25.00

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

Temperature	Time	Number of Cycles
95°C	15 min	1
98°C	1 min	3
72°C	1 min	
98°C	1 min	5
58°C	2 min	
60°C	4 min	
64°C	1 min	
72°C	1 min	
95°C	30 sec	18
66°C	3 min	
8°C	Hold	1

IMPORTANT: Precipitation may occur when the reactions are incubated at 8 °C overnight.

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

Gene-specific Primer Digestion

Hands-on time: 10-15 minutes

Total time: 50-55 minutes

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

1. Briefly spin the reactions to remove any droplets from the side walls. Carefully remove the seal or caps.
2. **Dilute Exonuclease:** 5 μ L of diluted exonuclease is needed per sample
 - a. Dilute 3 parts Exonuclease I with 2 parts 10x Exonuclease I buffer.
 - b. Mix by pipetting and place on ice.

Note: Exonuclease I is viscous. Ensure dilution is mixed thoroughly. Allow for overage when preparing dilution. Nuclease-free water can be used in place of 10x Exonuclease I buffer.

3. **Add Exonuclease:** Add 5 μ L of the diluted exonuclease to each sample, pipetting up and down to mix.
4. **Seal and mix:** Carefully seal the reactions. Pulse vortex the reactions on a medium setting for 5-10 seconds.
5. **Spin:** Briefly spin the reactions to remove any air bubbles from the bottom of the wells and spin down droplets from the seal or side walls.
6. **Perform digestion:** Perform the following program with the lid on:

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

IMPORTANT: Precipitation may occur when the reactions are incubated at 8°C overnight.

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

Gel Image After Gene-specific PCR and Primer Digestion

The following image is an example of samples after gene-specific PCR and primer digestion using various inputs of genomic DNA on a 2% agarose gel.

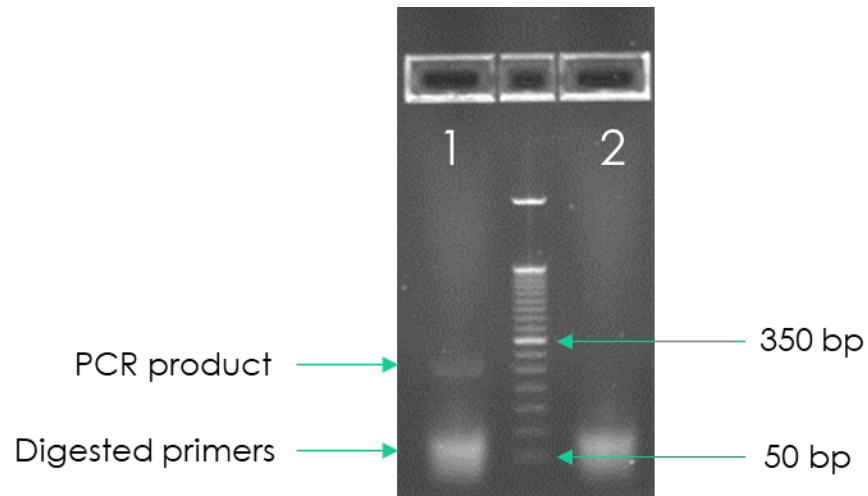


Figure 3. Gel analysis of GS PCR material. Lane 1 is genomic DNA samples and contains product after GS PCR and Exo digestion. Lane 2 is a no-template control.

Purify the Gene-specific PCR Product

Hands-on time: 30-45 minutes

Total time: 30-45 minutes

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

Pre-purification

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

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

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

Gene-specific Product Purification

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

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

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

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

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

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

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

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

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

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

12. **Resuspend beads:** Remove the samples from the magnetic rack, and immediately resuspend the dried beads in each well using 32 μ L nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly spin and mix again.
13. **Elute:** Incubate the elution at room temperature for 5 minutes to fully elute the product.
14. **Separate supernatant containing PCR product:** Place the samples on a magnetic rack until the solution appears clear, which can take up to 5 minutes.

TIP: After resuspending the beads, cover the samples and prepare the reactions for indexing the libraries using the Indexing PCR Master Mix in the

Pre-PCR area. Alternately, the purified gene-specific PCR product (on beads) may be stored at -20 °C after elution.

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

Indexing PCR: Amplify the Libraries

Hands-on time: 20-40 minutes

Total time: 50-70 minutes

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

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

Reagent	Volume (μL)
Pi700 Pillar Index	4.0
Pi500 Pillar Index	4.0
Total	8.0

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

Reagent	Volume (μL)
Indexing PCR Master Mix (2x)	25.0
GC Rescue D	5.0
Nuclease-free water	6.0
Total	36.0

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

Reagent	Volume (μL)
Pi500 and Pi700 Indices	8.0
Master Mix	36.0
Total	44.0

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

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

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

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

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

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

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

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

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

STOPPING POINT: The purified libraries may be stored at 4 $^{\circ}\text{C}$ for up to 3 days. Store the purified libraries at -20 $^{\circ}\text{C}$ for longer-term storage.

Final Library Image

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

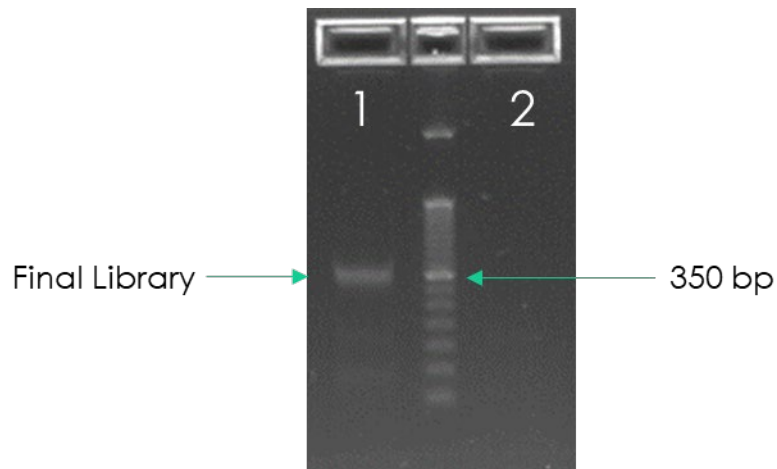


Figure 4. Gel analysis of final library material. Lane 1 is a genomic DNA sample and contains product after indexing and cleanup. Lane 2 is a no-template control.

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 Qubit working solution:** 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 working solution from light.*

2. **Label tubes:** Set up 0.5 mL Qubit tubes for standards and samples. Label the tube lids.
3. **Prepare standards:** Transfer 190 μL of Qubit working solution into two tubes for standard 1 and standard 2, and then add 10 μL of each standard to the corresponding tube.
4. **Prepare samples:** Transfer 198 μL of Qubit working solution to each tube, and then add 2 μL of each sample to the tube (1:100 dilution).
5. **Mix and spin:** Mix the tubes by vortexing and then spinning the tubes briefly.
6. Incubate the tubes at room temperature for 2 minutes.
7. **Measure concentration:** Measure the concentration of each sample on the Qubit 2.0 Fluorometer per the Qubit User Guide. Use the dsDNA High Sensitivity assay to read standards 1 and 2 followed by the samples.
 - a. If any sample concentrations are above the linear range of the instrument, prepare a new dilution using 199 μL Qubit working solution and 1 μL sample (1:200 dilution). Repeat steps 5-7.
8. **Calculate concentration:** 1 ng/ μL of library is equal to **5 nM**. Example calculation is below. Adjust dilution factor accordingly.

2 uL of library + 198 uL Qubit working solution:

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

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

Normalization and Pooling

Hands-on time: 30-45 minutes

Total time: 30-45 minutes

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

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

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

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

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

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

Prepare Diluted Libraries for Sequencing

Hands-on time: 30-70 minutes

Total time: 30-70 minutes

Samples can be multiplexed and sequenced on the MiSeq using the v3 chemistry or the NextSeq using a Mid kit. The number of samples that can be loaded is dependent on the number of paired-end reads per sample, and is displayed in the table below. Please choose the appropriate sequencing workflow and kit based on the number of samples to be sequenced.

Kit	Cycles	Estimated PE reads	Estimated PE reads/sample	Estimated maximum # libraries
MiSeq v2	2x150	30 Million	3.7 Million	8
MiSeq v3	2x150	50 Million	3.5 Million	14
	2x275	50 Million	3.5 Million	14
NextSeq Mid	2x150	260 Million	3.5 Million	96

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

Sequencing on MiSeq (MiSeq v2 or v3 Kit)

For v2 or v3 chemistry (MiSeq v2 or v3 kit), dilute libraries to **5 nM**. The final concentration of the libraries for sequencing is **10 pM**.

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

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

Alternately, prepare a 1 N NaOH solution by combining 500 μ L 10 N NaOH into 4.5 mL of nuclease-free water. Vortex the solution to mix. If 1 N NaOH has not been prepared within the last week from a 10 N solution, prepare a new 1 N NaOH solution.

2. **Denature the library mix:** Label a new 1.5 mL microtube for the denatured, 25 pM library mix.

- a. Denature the library mix by combining 5 μL of the library mix and 5 μL of the freshly prepared 0.2 N NaOH.
 - b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
 - c. Let the solution stand at room temperature for 5 minutes.
 - d. Add 990 μL of Illumina's HT1 solution to the denatured library mix.
 - e. Invert the mixture several times, spin briefly, and place on ice.
3. **Dilute to 10 pM library mix:** Label a new 1.5 mL microtube for the 10 pM library mix. Combine 240 μL of the 25 pM library mix (step 5) with 360 μL of Illumina's HT1 solution. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, spin briefly, and place on ice
 4. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 594 μL of the 10 pM library mix (step 6) with 6 μL of a 20 pM PhiX library control. Briefly vortex, spin, and place on ice.
 5. **Load MiSeq cartridge:** Using a clean 1000 μL tip, puncture the foil cap above the sample loading tube on the MiSeq cartridge. Load the 600 μL library mix and PhiX mixture (step 7) into the cartridge and ensure the solution has reached the bottom of the tube by lightly tapping the tube if liquid remains on the side wall or if there is an air bubble at the bottom of the tube.
 6. **Run the MiSeq:** Run the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) and two indexing reads of 8 cycles each: "MiSeq System User Guide" (part #15027617). For instructions on preparing a sample sheet for the MiSeq, see page 29.
 7. Store diluted libraries and mixtures at -20°C for long-term storage.

Sequencing on the NextSeq

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

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

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

Alternately, prepare a 1 N NaOH solution by combining 500 μL 10 N NaOH into 4.5 mL of nuclease-free water. Vortex the solution to mix. If 1 N NaOH has not been prepared within the last week from a 10 N solution, prepare a new 1 N NaOH solution.

2. **Denature the library mix:** Label a new microtube for the denatured, 25 pM library mix.
 - a. Denature the library mix by combining 5 μL of the library mix and 5 μL of the freshly prepared 0.2 N NaOH.
 - b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
 - c. Let the solution stand at room temperature for 5 minutes.
 - d. Add 5 μL of 200 mM Tris-HCl, pH 7.0.
 - e. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
 - f. Add 985 μL of Illumina's HT1 solution to the denatured library mix.
 - g. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
3. **Dilute 25 pM library mix to 1.8 pM:** Dilute the denatured library to 1400 μL of a 1.8 pM solution by combining 101 μL of the 25 pM denatured library mix with 1299 μL of Illumina's HT1 solution. Invert to mix and spin briefly.
4. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 1287 μL of the 1.8 pM library mix (step 6) with 13 μL of a 1.8 pM PhiX library control. Briefly vortex, spin, and place on ice.
5. **Load NextSeq cartridge:** Using a clean 1000 μL tip, puncture the foil cap above the sample loading well on the NextSeq cartridge. Load 1300 μL library mix and PhiX mixture (step 7) into the cartridge and ensure the solution has reached the bottom of the cartridge well.

6. **Run the NextSeq:** Run the libraries on the NextSeq per the manufacturer's instructions using a paired-end read length of 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 29.
7. **Store Libraries:** Store diluted libraries and mixtures at -20°C for long-term storage.

Preparing a Sample Sheet for Sequencing

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

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

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

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

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

TROUBLESHOOTING

Issue	Potential Cause	Solution
Low yield of gene-specific product	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 samples.
	Improper cycling	Check that the cycling protocol performed is the appropriate protocol for gene-specific amplification.
Low indexing efficiency	Improper AMPure purification	Incomplete AMPure purification or loss of gene-specific product will affect the indexing PCR reaction. The purified product can be checked on an agarose gel to ensure the gene-specific product was not lost or that clean-up was sufficient to remove excess primers.
		The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.
		Leftover ethanol from the wash steps can hinder the PCR reaction. Remove as much of the ethanol during the final wash step with a pipette and dry the beads to ensure the residual ethanol has evaporated.
	Partial primer digestion	Poor digestion of the gene-specific primers can hinder the indexing efficiency of the indexing PCR reaction. Check the primer digestion using an agarose gel.
	Incomplete deactivation of exonuclease	The inactivation of the nuclease and AMPure purification is necessary before performing indexing PCR. Leftover active exonuclease can

		digest the indexing PCR primers, reducing the yield of the indexing PCR reaction.
Low library yield	DNA quantity or quality	<p>The recommended input for the assay is 20-60ng of genomic DNA. Higher quantities may be necessary for low or poor quality FFPE samples.</p> <p>Run the product from the gene-specific PCR on agarose gel to check the yield.</p> <p>The product can also be checked on an agarose gel after indexing PCR before and after AMPure purification.</p>
	Improper AMPure purification	<p>Incomplete AMPure purification or loss of product will affect the final yield. The purified product can be checked on an agarose gel to ensure the product was not lost during PCR cleanup.</p> <p>The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash.</p>
The libraries over-cluster or under-cluster on the MiSeq	Normalization and mix of libraries is not 25 pM (MiSeq), or 1.8 pM (NextSeq)	Check the 5 nM library mix using Qubit or RT-PCR. Dilute the denatured library mix as needed to adjust for the difference in concentration.
	Improper library Quantitation	<p>Improper library quantitation may result in artificially high or low yields, which affects downstream normalization.</p> <p>Re-quantitate the final libraries and/or the normalized libraries to check for the expected values.</p>

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