INHERIT/Reveal™ Thalassemia Panel

Library Preparation User Guide

FOR RESEARCH USE ONLY

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Pillar Biosciences
pillar-biosciences.com
Version 2.3

UM-0009

INTRODUCTION

The INHERIT/Reveal Thalassemia Panel targets numerous, relevant gene regions of interest for researchers looking to explore the genetic sequences of germline DNA samples from α-Thalessemia and β-Thalessemia cases. The panel utilizes Pillar Biosciences' proprietary SLIMamp® (stem-loop inhibition mediated amplification) technology, allowing researchers to amplify regions of interest 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 INHERIT/Reveal Thalassemia 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 INHERIT/Reveal Thalassemia 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).

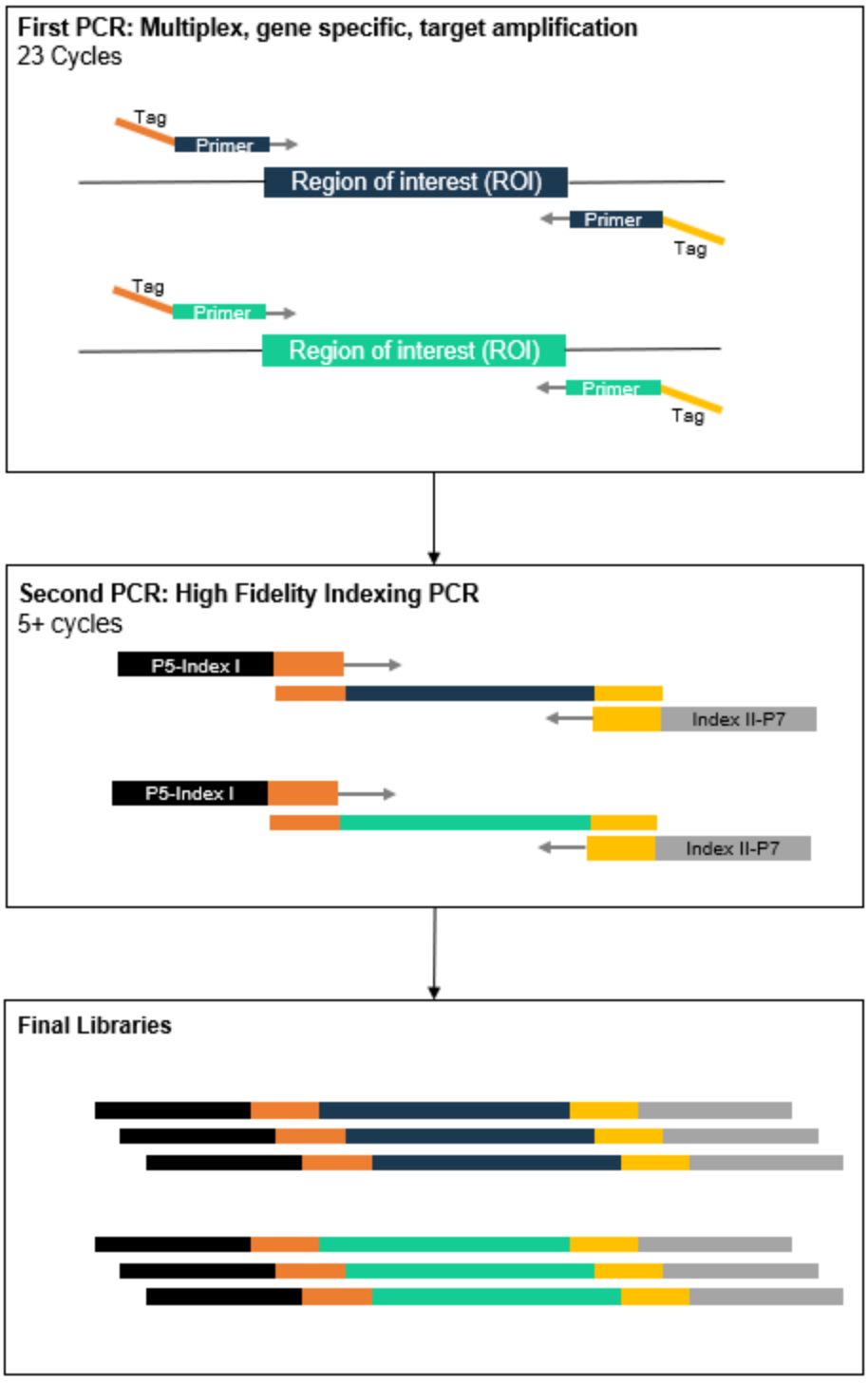


Figure 1. Overview of INHERIT/Reveal Thalassemia Panel library preparation.

REVISION HISTORY

2019-01: Initial Release
2019-06: Update for v2 pool
2019-11: Formatting and branding
2020-07: Added Sample Set-Up and PiVAT Analysis sections in version 2.3

GETTING STARTED

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

Components of the INHERIT/Reveal Thalassemia Panel

Reagent	Use	Area Use	Storage
Thalassemia v2 Oligo Pool	Gene-specific PCR	Pre-PCR	-15° to -25°C
Gene Specific PCR Master Mix (2x)	Gene-specific PCR	Pre-PCR	-15° to -25°C
Indexing PCR Master Mix (2x)	Indexing PCR	Pre-PCR	-15° to -25°C

All reagents in the kit should be used in designated areas specified above to prevent amplicon contamination.

INHERIT/Reveal Thalassemia Panel Indexing Kits

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

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

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
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)	Post-PCR	Teknova, Cat#T7724

Compatible Illumina Reagent Kits

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
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, Part Number 6551 or equivalent
Microplate sealing film	Pre- and post-PCR	Axygen, Part Number 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, Part Number 13-681-506 or equivalent
Qubit Assay tubes	Post-PCR	Invitrogen, Part Number 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 (including refrigerators, freezers) 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.

INHERIT/Reveal Thalassemia Panel WORKFLOW

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

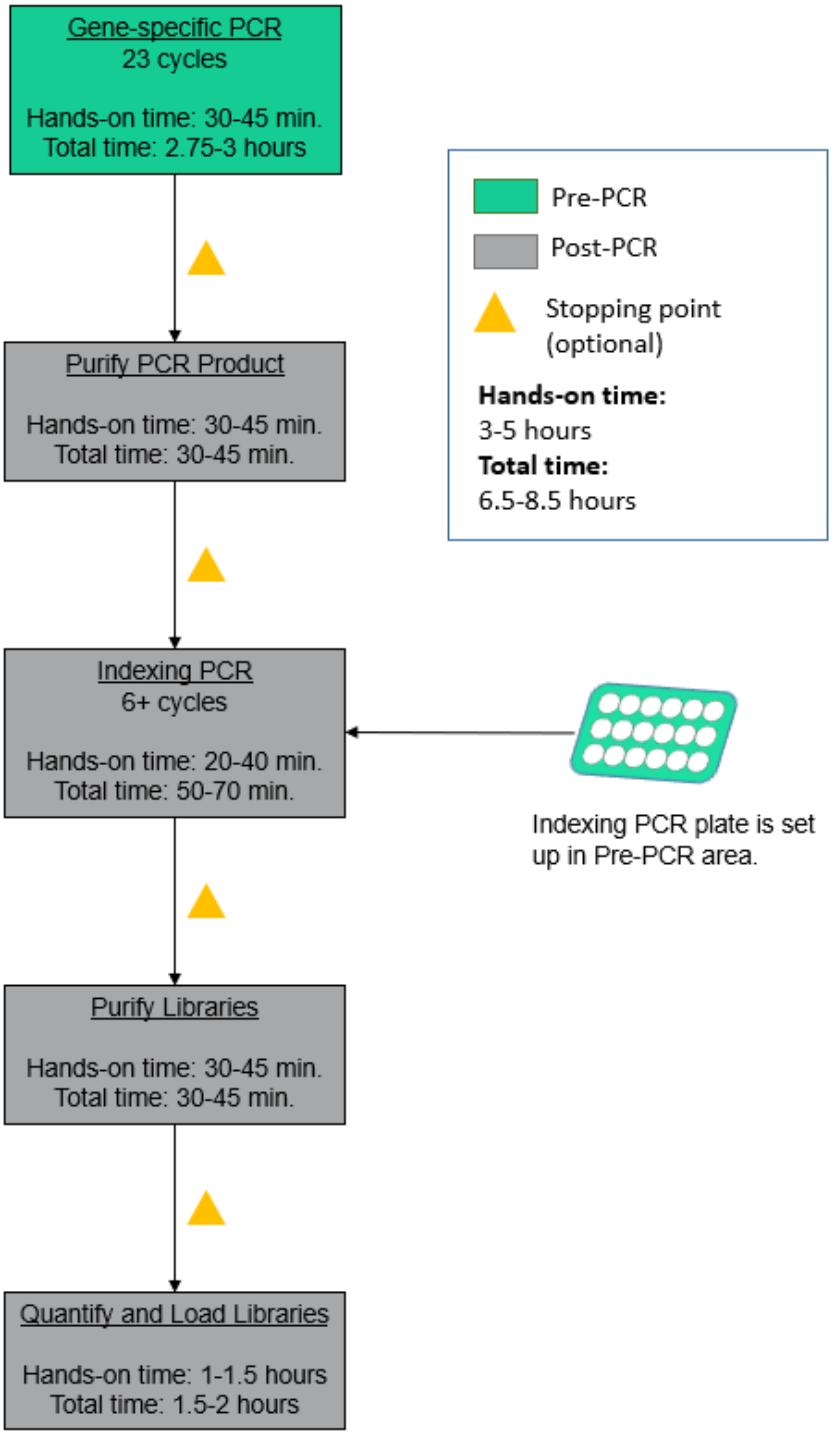


Figure 2. The INHERIT/Reveal Thalassemia Panel workflow can be completed within a day but contains multiple stopping points for users with time constraints.

Sample Set-Up

The Thalassemia analysis is based on double normalization method, including one per-sample normalization and one per-amplicon normalization. The normalization baseline is calculated from negative reference samples. The copy number variance (CNV) is detected by amplicon clusters and the Thalassemia type of each sample is called by matching the edges of a CNV with the edges of a list of common Thalassemia types.

For each Thalassemia analysis run, the user should provide 3-5 (minimum 2) in-run normal (negative) reference samples with similar sample condition and preparation process as the positive samples. If less than 2 negative reference samples are provided, the run will fail.

DNA INPUT INFORMATION

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

The recommended DNA input is 10-75 ng per PCR reaction for standard genomic DNA. For good quality gDNA samples that are not degraded, as little as 5 ng of input may be used with acceptable yield and mapping rate. For gDNA samples of low quality that may be degraded, it is recommended that at least 20 ng of input be used. However, if it is necessary, low input amount of low-quality DNA samples may be used, but the library yield may be low (< 5 nM) and the mapping rate may be low (< 90%). If lower input outside of the recommended input range is being used, it is recommended that at least 6 cycles of indexing be performed.

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 Master Mix (GS PCR MMX) and INHERIT/Reveal Thalassemia Panel Oligo Pool on ice.

1. **Dilute input DNA:** Dilute DNA in nuclease-free water to a final volume of 7.5 µL. Add 7.5 µL of nuclease-free water to the no-template control (NTC) well.

Reagent	Volume (µL)
Diluted DNA* (or water)	7.5
Total	**7.5**

**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 range is 10-75 ng per PCR reaction for standard genomic DNA. It is recommended that the quality of DNA be checked by qPCR (Taqman RNase P Detection Reagents Kit (Life Technologies, Cat. No. 4316831)) or a Bio-analyzer. If the DNA is not severely degraded, the DNA input can be as little as 10 ng. However, if the DNA is severely degraded, it is recommended to increase the DNA input quantified by qPCR or Bio-analyzer when possible. See "DNA Input Information" for guidelines on DNA input.

2. **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.

> ***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. Prepare the PCR master mix. Volumes are calculated on a per sample basis. Allow for overage when preparing PCR master mix for multiple samples. Include a no-template control when calculating total number of samples.

Reagent	Volume (µL)
Gene-specific PCR Master Mix	12.5
Thalassemia oligo pool	5.0
Total	**17.5**

b. Mix the PCR master mix by vortexing on medium speed for 10 seconds and spin down the solution in a microfuge. Transfer 17.5 µL of master mix to each sample well in a PCR plate, strip tube, or PCR tube.

Reagent	Volume (µL)
Gene-specific PCR Master Mix	12.5
Thalassemia oligo pool	5.0
Diluted DNA* (or water)	7.5
Total	**25.0**

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

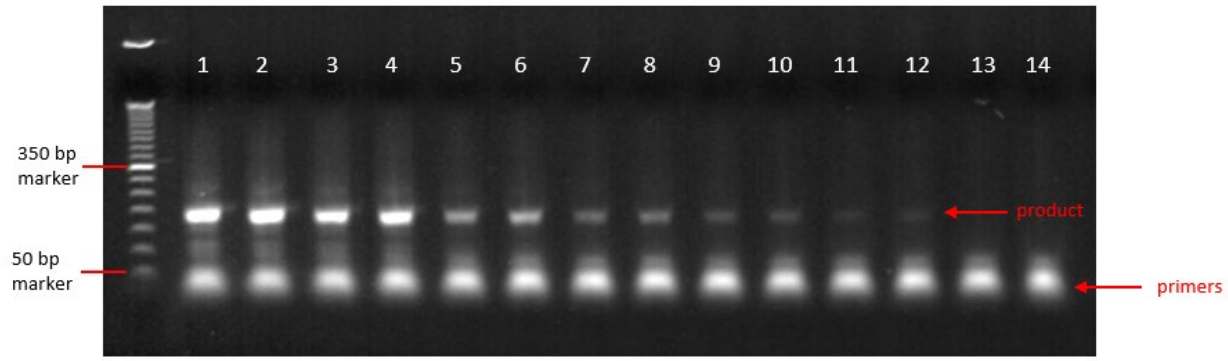
Temperature	Time	Number of Cycles
95°C	15 minutes	1
98°C 58°C 60°C 64°C 72°C	1 minute 2 minutes 4 minutes 1 minute 1 minute	5
95°C 66°C	30 seconds 3 minutes	18
8°C	Hold	1

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.*

Quality Control

The following image is an example of genomic DNA samples after the Gene Specific PCR using various inputs on a 2% agarose gel.



Genomic DNA: lanes 1-12

No template control (NTC): lanes 13-14

Input amount: 75 ng (lanes 1-2), 50 ng (lanes 3-4), 20 ng (lanes 5-6), 10 ng (lanes 7-8), 5 ng (lanes 9-10), 2.5 ng (lanes 11-12).

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 and 70% ethanol.*

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 dislodging 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.

1. **Dry beads:** Let the beads air dry at room 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.*

11. **Resuspend beads:** Remove the samples from the magnetic rack, and immediately resuspend the 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 and mix again.

    ***Note:** Do not allow the beads to over-dry. During the purification, the beads tend to clump and "fan." Be sure to immediately resuspend the beads after removing the ethanol wash.*

12. 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.

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 and water sufficient for the number of samples being processed with overage.

Reagent	Volume (µL)
Indexing PCR Master Mix (2x)	25.0
Nuclease-free water	11.0
Total	**36.0**

3. **Add master mix to wells:** Add prepared master mix to wells that contain indices from step 1. 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.
4. **Add gene-specific PCR product:** The following steps should be performed in a post-PCR area. ***Important***: Cover or seal the reactions before transferring from the pre-PCR area to the post-PCR area.

   Add 6 µL of the gene-specific PCR product, without the beads, to the corresponding wells and carefully seal the plate or cap the tubes. Small amounts of bead carry-over may occur and will not significantly impact the PCR reaction.

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

5. **Mix and spin:** Pulse vortex the sealed reactions on a medium setting for 5-10 seconds to mix. Briefly spin down the reactions to remove any bubbles within the reaction solutions.
6. **Perform PCR:** Perform the following program with the heated lid on:

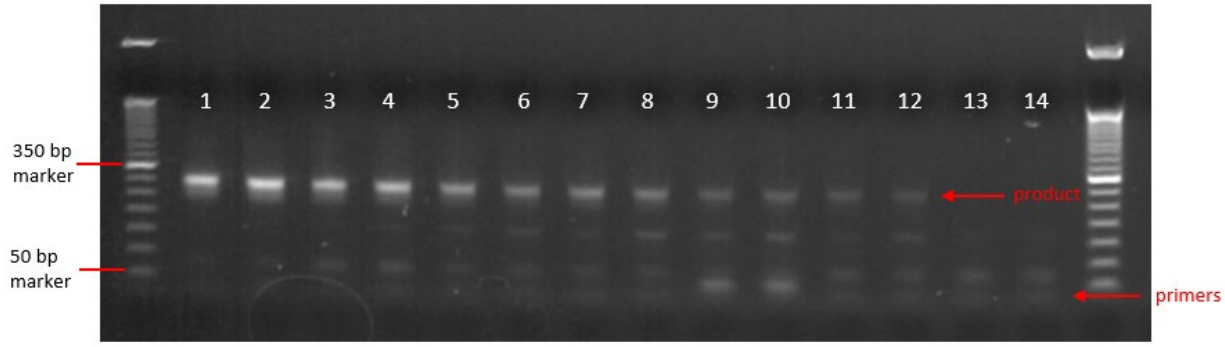
Temperature	Time	Number of Cycles
95°C	2 min	1
95°C 66°C 72°C	30 sec 30 sec 60 sec	6*
72°C	5 min	1
8°C	Hold	1

**The number of indexing cycles is dependent on starting input amount final library yield. If starting with the recommended input range of DNA, 6 cycles of indexing is recommended. If using lower than the recommended range of input DNA, then additional cycles may be needed to achieve sufficient library yield.*

STOPPING POINT: *The indexed libraries may be stored at -20°C.*
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 27 for the MiSeq or page 29 for the NextSeq).*

Quality Control

The following image is an example of libraries after 5 cycles of indexing PCR on a 2% agarose gel. Note that samples in lanes 1-8 use DNA input amounts within the recommended input range. Samples in lanes 9-12 use DNA input amounts lower than 10 ng and produce fainter bands. At least 6 cycles of indexing should be performed for samples with DNA input amounts below 10 ng.



Genomic DNA: lanes 1-12

No template control (NTC): lanes 13-14

Input amount: 75 ng (lanes 1-2), 50 ng (lanes 3-4), 20 ng (lanes 5-6), 10 ng (lanes 7-8), 5 ng (lanes 9-10), 2.5 ng (lanes 11-12).

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 the volume is not currently 50 uL) 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 dislodging 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*

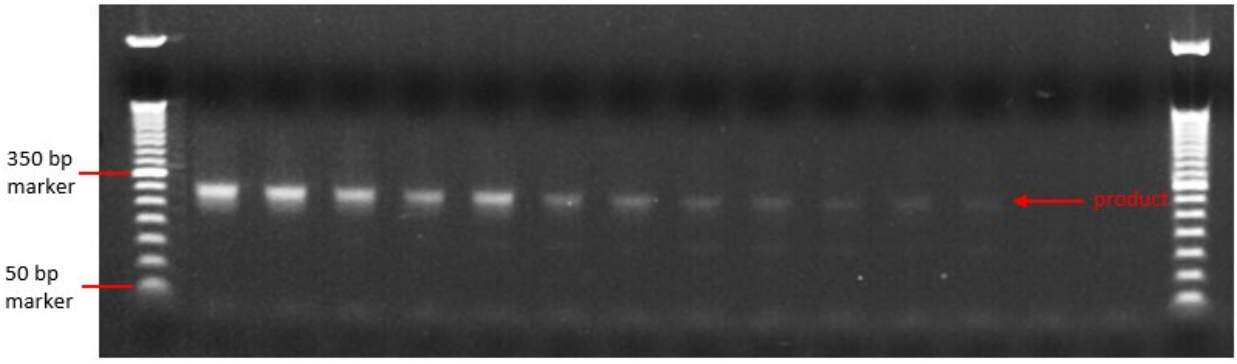
section. The purified libraries may also be stored at 4℃ for up to 3 days or at -20℃ for longer-term storage.

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.*

Quality Control

The following image is an example of final libraries after purification on a 2% agarose gel. Note that samples in lanes 1-8 use DNA input amounts within the recommended input range. For samples in lanes 9-12, DNA input amounts of lower than 10 ng were used, and faint product bands formed after 5 cycles of indexing. At least 6 cycles of indexing should be performed for samples with DNA input amounts below 10 ng.



Genomic DNA: lanes 1-12

No template control (NTC): lanes 13-14

Input amount: 75 ng (lanes 1-2), 50 ng (lanes 3-4), 20 ng (lanes 5-6), 10 ng (lanes 7-8), 5 ng (lanes 9-10), 2.5 ng (lanes 11-12).

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:** 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 solution:

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

   ***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, MiSeq v3 chemistry, or a NextSeq. 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 INHERIT/Reveal Thalassemia 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. As an example, when using v3 chemistry on a MiSeq, a similar number of PE reads can be obtained with a (A) cluster density of approximately 1100 K/mm^2 and a passing filter of 94% or (B) cluster density of approximately 1300 K/mm^2 and a passing filter of 85%. Therefore, the estimates below are representative of libraries that obtain a cluster density from 830-1100 K/mm^2 and a passing filter >88% for v2 chemistry and 1100-1600 K/mm^2 with a passing filter >85% for v3 chemistry.

Kit	Cycles	Estimated PE reads	Est. Mean amplicon coverage (x)[a]	Est. Min. amplicon coverage (x)[b]	Estimated PE reads/sample	Est. Maximum # libraries
MiSeq Nano v2	2x150	2 Million	1000	200	333,300	6
MiSeq Micro v2	2x150	8 Million	1000	200	296,200	27
MiSeq v2	2x150	30 Million	1000	200	291,200	103
MiSeq v3	2x150	50 Million	1000	200	289,000	173
NextSeq Mid	2x150	260 Million	12700	200	288,900	900

[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.
The number of libraries loaded onto sequencer is limited by the unique index combinations.

Normalize Libraries to 5 nM

1. **Dilute Libraries:** 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. Follow the example calculation below:

$$\frac{Library\ concentration\ (nM)\ x\ 4\ uL\ library}{5\ nM} = Final\ volume\ of\ library$$

$$Final\ volume\ of\ library - 4\ uL\ library = volume\ of\ diluent$$

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.

   The libraries prepared using the INHERIT/Reveal Thalassemia Panel cluster very efficiently on the MiSeq. It is recommended that the library mix be quantified using Qubit or another library quantification 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 3 accordingly to obtain the desired concentration.

Sequencing using v2 chemistry (MiSeq Nano v2, MiSeq Micro v2 or MiSeq v2 kit)
For running a MiSeq kit using v2 chemistry (MiSeq Micro v2 or MiSeq v2 kits), the recommended 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 µL nuclease-free water with 200 µL of 1 N NaOH. Vortex the solution to mix.

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

2. **Denature the library mix:** Label a new microtube for the denatured, 25 pM library mix.
   a. Denature the library mix by combining 5 µL of the library mix and 5 µL of the freshly prepared 0.2 N NaOH.
   b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
   c. Let the solution stand at room temperature for 5 minutes.
   d. Add 990 µL of Illumina's HT1 solution to the denatured library mix.
   e. Invert the mixture several times, spin briefly, and place on ice.
3. **Dilute to 10 pM library mix:** Label a new 1.5 mL microtube for the 10 pM library mix. Combine 240 µL of the 25 pM library mix (step 2) with 360 µL of Illumina's HT1 solution. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, spin briefly, and place on ice.
4. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 594 µL of the 10 pM library mix (step 3) with 6 µL of a 12.5 pM PhiX library control. Briefly vortex, spin, and place on ice.
5. **Load MiSeq cartridge:** Using a clean 1000 µL tip, puncture the foil cap above the sample loading tube on the MiSeq cartridge. Load the 600 µL library mix and PhiX mixture (step 4) into the cartridge and ensure the solution has reached the bottom of the tube by lightly tapping the tube if liquid remains on the side wall or there is an air bubble at the bottom of the tube.
6. **Run the MiSeq:** Run the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150): "MiSeq System User Guide" (part #15027617).
7. 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), the final concentration of the libraries for sequencing is **12.5 pM.**

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

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

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

2. **Denature the library mix:** Label a new 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 12.5 pM library mix:** Label a new 1.5 mL microtube for the 10 pM library mix. Combine 300 µL of the 25 pM library mix (step 2) with 300 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 12.5 pM library mix (step 2) with 6 µL of a 12.5 pM PhiX library control. Briefly vortex, spin, and place on ice.
5. **Load MiSeq cartridge:** Using a clean 1000 µL tip, puncture the foil cap above the sample loading tube on the MiSeq cartridge. Load the 600 µL library mix and PhiX mixture (step 3) into the cartridge and ensure the solution has reached the bottom of the tube by lightly tapping the tube if liquid remains on the side wall or there is an air bubble at the bottom of the tube.
6. **Run the MiSeq:** Run the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150): "MiSeq System User Guide" (part #15027617).
7. Store diluted libraries and mixtures at -20°C for long-term storage.

Sequencing on the NextSeq

For sequencing on the NextSeq, the recommended final concentration of the libraries for sequencing is **1.8 pM**.

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

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

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

2. **Denature the library mix:** Label a new microtube for the denatured, 25 pM library mix.
   a. Denature the library mix by combining 5 µL of the library mix and 5 µL of the freshly prepared 0.2 N NaOH.
   b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
   c. Let the solution stand at room temperature for 5 minutes.
   d. Add 5 µL of 200 mM Tris-HCl, pH 7.0.
   e. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
   f. Add 985 µL of Illumina's HT1 solution to the denatured library mix.
   g. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
3. **Dilute 25 pM library mix to 1.8 pM:** Dilute the denatured library to 1400 µL of a 1.8 pM solution by combining 101 µL of the 25 pM denatured library mix with 1299 µL of Illumina's HT1 solution. Invert to mix and spin briefly.
4. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 1287 µL of the 1.8 pM library mix (step 3) with 13 µL of a 1.8 pM PhiX library control. Briefly vortex, spin, and place on ice.
5. **Load NextSeq cartridge:** Using a clean 1000 µL tip, puncture the foil cap above the sample loading well on the NextSeq cartridge. Load the 1300 µL library mix and PhiX mixture (step 4) into the cartridge and ensure the solution has reached the bottom of the cartridge well.
6. **Run the NextSeq:** Run the libraries on the NextSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) and two indexing reads of 8 cycles each: "NextSeq System User Guide" (part #15046563 or 15069765).
7. Store diluted libraries and mixtures at -20°C for long-term storage.

Preparing a Sample Sheet

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. If samples prepared using the INHERIT/Reveal INHERIT/Reveal Thalassemia Panel are being loaded with samples prepared with the TSCA kit, ensure that the indices from both sets of samples have unique barcode sequences.

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 choose "MiSeq" or "NextSeq" sample sheet according to which sequencing instrument is being loaded. 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/or 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.

Once all requirements for the sample sheet are met, export the sample sheet as a comma-separated values (.csv) file by clicking "Export".

PiVAT Analysis

See the *PiVAT Analysis Manual* for instructions on starting analysis following a Thalassemia run. The THAL_RESULTS excel file is output upon completion of the analysis. The following is an explanation of the results contained in the THAL_RESULTS file.

PiVAT 2020.1 uses two different algorithms to call Thalassemia separately: a machine learning based "Thal General Caller" and a CNV edge matching based "Thal Type Caller". In the *THAL Labels* sheet of the THAL_RESULTS excel file, two binary (TRUE/FALSE) labels from both callers are listed for each sample. Calls made by each caller are further detailed in two separate sheets:

1. Detailed Thalassemia call information from "Thal Type Caller" are in the *THAL Type Calls* sheet of the THAL_RESULTS excel file and calls from "Thal General Caller" are in the *THAL General Calls* sheet.
2. In the *THAL General Calls* sheet, the detected CNV amplicon clusters on chromosome 11 and chromosome 16 are outputted for each sample labeled "TRUE" by the "Thal General Caller".

In general, "Thal Type Caller" gives more accurate and reliable results and the *THAL Type Calls* sheet is recommended to be used as the major reference for Thalassemia calls.

In the *THAL Labels* sheet, if **Thal_Type_Label** is FALSE but **Thal_General_Label** is TRUE for a sample, the user can refer to the CNV information in *the THAL General Calls* sheet and check if any detected CNV is related to Thalassemia. The fully normalized copy numbers can be found in the *Fully Normalized* sheet in the THAL_RESULTS excel file. Note that the copy number in PiVAT is defined as the copy number ratio between a sample and the negative reference (diploid with 2 copy number).

<u>List of user adjustable sample Quality Control (QC) parameters:</u>

1. Negative reference QC:
    a. CNV_QC_ABSOLUTE_COVERAGE_THRESHOLD_NEG_SAMPLE >= 50
    b. CNV_QC_Q30_MAPPING_RATE_THRESHOLD >= 80%
    c. CNV_QC_ON_TARGET_RATE_THRESHOLD >= 90%
    d. CNV_QC_CENTER_CORRELATION_COEFFICIENT_THRESHOLD >= 0.50
2. Positive sample QC:
    a. CNV_QC_ABSOLUTE_COVERAGE_THRESHOLD_POS_SAMPLE >= 10
    b. CNV_QC_Q30_MAPPING_RATE_THRESHOLD >= 80%
    c. CNV_QC_ON_TARGET_RATE_THRESHOLD >= 90%
    d. CNV_SAMPLE_OUTLIER_CORRELATION_THRESHOLD >= 0.20

(Users may ask admin to change these parameters if needed.)

In the following tables, the column headers in the calls sheets of the THAL_RESULTS file are explained.

"THAL Labels" Sheet Column Explanation:

Sample_ID	Unique Sample ID for each sample.
Thal_Type_Label	TRUE or FALSE label from "Thal Type Caller".
Thal_General_Label	TRUE or FALSE label from "Thal General Caller".

"THAL Type Calls" Sheet Column Explanation:

Sample_ID	Unique Sample ID for each sample
THAL_TYPE	The Thalassemia type detected by the "Thal Type Caller" for a sample.
THAL_CHROM	The chromosome where the thalassemia is detected.
THAL_START	The reported (referenced) start position of the **THAL_TYPE**.
THAL_END	The reported (referenced) end position of the **THAL_TYPE**.
THAL_CNV	Mean of normalized copy number ratio of the amplicons in the called thalassemia CNV.
ZScore	Calculated Z-score of the thalassemia CNV.
CNV_Start	The location on **THAL_CHROM** where the thalassemia CNV starts. A range is reported based on the amplion lengths.
CNV_End	The location on **THAL_CHROM** where the thalassemia CNV ends. A range is reported based on the amplion lengths.
THAL_TARGET_INDEX	List of amplicon indices in the called thalassemia CNV.
THAL_TARGET_IDS	List of amplicon names in the called thalassemia CNV.

"THAL General Calls" Sheet Column Explanation:

Sample_ID	Unique Sample ID for each sample
Thal_General_Label	The TRUE/FALSE from "Thal General Caller" for the sample.
Cov_Norm_List	List of normalized copy number ratios of the amplicons in the called CNV amplicon cluster.
Amp_Ct	Count of amplicons in the called CNV amplicon cluster.
Target_Name	List of the amplicon names in the called CNV amplicon cluster.
Cov_Norm_Mean	Mean of the normalized copy number ratio for the amplicons in the called CNV amplicon cluster.
chromosome	The chromosome where the CNV is detected.
ZScore	Calculated Z-score of the reported CNV.
Gene_Symbol	Simplified gene names for the amplicons in the reported CNV amplicon cluster.
Cov_Norm_Std	Standard deviation calculated based on the copy number ratios of the amplicons in the reported CNV amplicon cluster.
start_pos	The location on **chromosome** where the cluster CNV starts. This is the start of the first amplicon in the CNV amplicon cluster.
CNV_Call	Average copy number ratio of the called CNV amplicon cluster. This is calculated by taking the ratio between copy number of the sample and copy number (which is 2) of the negative references.
end_pos	The location on **chromosome** where the cluster CNV ends. This is the end of the last amplicon in the CNV amplicon cluster.

TROUBLESHOOTING

Issue	Potential Cause	Solution
Low yield of gene-specific product (GS product not visible at all on gel or equivalent)	DNA quantity or quality	The recommended input for the assay is 10-75 ng of genomic DNA. Higher quantities may be necessary for low 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	The recommended input for the assay is 10-75 ng 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), 10 pM (v3), 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 quantification	Improper library quantification may result in artificially high or low yields, which affects downstream normalization. Re-quantify 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. 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.

i7 Index	
Index Name	Index Barcode Sequence
Pi701	ATTACTCG
Pi702	TCCGGAGA
Pi703	CGCTCATT
Pi704	GAGATTCC
Pi705	ATTCAGAA
Pi706	GAATTCGT
Pi707	CTGAAGCT
Pi708	TAATGCGC
Pi709	ATCACGAC
Pi710	ACAGTGGT
Pi711	CAGATCCA
Pi712	ACAAACGG
Pi713	GAAACCCA
Pi714	TGTGACCA
Pi715	AGGGTCAA
Pi716	AGGAGTGG

i5 Index		
Index Name	Index Barcode Sequence for Miseq	Index Barcode Sequence for NextSeq
Pi501	TATAGCCT	AGGCTATA
Pi502	ATAGAGGC	GCCTCTAT
Pi503	CCTATCCT	AGGATAGG
Pi504	GGCTCTGA	TCAGAGCC
Pi505	AGGCGAAG	CTTCGCCT
Pi506	TAATCTTA	TAAGATTA
Pi507	CAGGACGT	ACGTCCTG
Pi508	GTACTGAC	GTCAGTAC
Pi509	TGAACCTT	AAGGTTCA
Pi510	TGCTAAGT	ACTTAGCA
Pi511	TGTTCTCT	AGAGAACA
Pi512	TAAGACAC	GTGTCTTA
Pi513	CTAATCGA	TCGATTAG
Pi514	CTAGAACA	TGTTCTAG
Pi515	TAAGTTCC	GGAACTTA
Pi516	TAGACCTA	TAGGTCTA