

# inheritReveal™ Thalassemia

## USER MANUAL



## **Revision History**

Version 1.0: Initial Release

Version 2.0: Overhauled formatting, branding, and contents

# Table of Contents

<b>I.</b>	<b>PRODUCT INTRODUCTION.....</b>	<b>3</b>
<b>2.</b>	<b>PRODUCT DESCRIPTION.....</b>	<b>3</b>
<b>3.</b>	<b>GENERAL LABORATORY GUIDELINES.....</b>	<b>4</b>
<b>4.</b>	<b>REAGENTS &amp; EQUIPMENT.....</b>	<b>5</b>
4.1	Kit Components.....	5
4.2	Indexing Kits.....	5
4.3	User-Supplied Reagents.....	5
4.4	Other Consumables.....	6
4.5	Equipment.....	7
<b>5.</b>	<b>WORKFLOW.....</b>	<b>8</b>
<b>6.</b>	<b>DNA INPUT INFORMATION.....</b>	<b>9</b>
<b>7.</b>	<b>LIBRARY PREPARATION PROTOCOL.....</b>	<b>10</b>
7.1	Gene-Specific PCR (GS-PCR) & Purification.....	10
7.2	Index PCR & Purification.....	14
7.3	Quantitation and Normalization of Purified Libraries.....	18
<b>8.</b>	<b>SEQUENCING.....</b>	<b>21</b>
<b>9.</b>	<b>TROUBLESHOOTING LIBRARY PREPARATION.....</b>	<b>26</b>
<b>10.</b>	<b>APPENDIX A: INDEX SEQUENCES.....</b>	<b>29</b>
<b>11.</b>	<b>APPENDIX B: THALASSEMIA ANALYSIS USING PIVAT®.....</b>	<b>30</b>

## 1. Product Introduction

The **inheritReveal™ Thalassemia Panel** is a robust NGS assay that targets numerous gene regions of interest for researchers looking to explore the genetic sequences of germline DNA samples from  $\alpha$ -Thalassemia and  $\beta$ -Thalassemia cases. The inheritReveal™ Thalassemia Panel utilizes our SLIMamp® (stem-loop inhibition mediated amplification) technology with a single-tube multiplex PCR reaction. The panel requires a unique dual barcode indexing kit, available separately, and the resulting libraries are designed for sequencing on the Illumina platform using a paired-end read length of 2x150. The workflow of this panel can be performed and loaded onto the sequencing instrument within one day. In addition, the protocol also contains numerous stopping points for users who have time limitations.

## 2. Product Description

Pairs of DNA oligos designed for each region of interest, or hotspot, 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 & P7 sequences to each library for sample tracking and sequencing. The final libraries are further purified and can be sequenced on the Illumina sequencing platform.

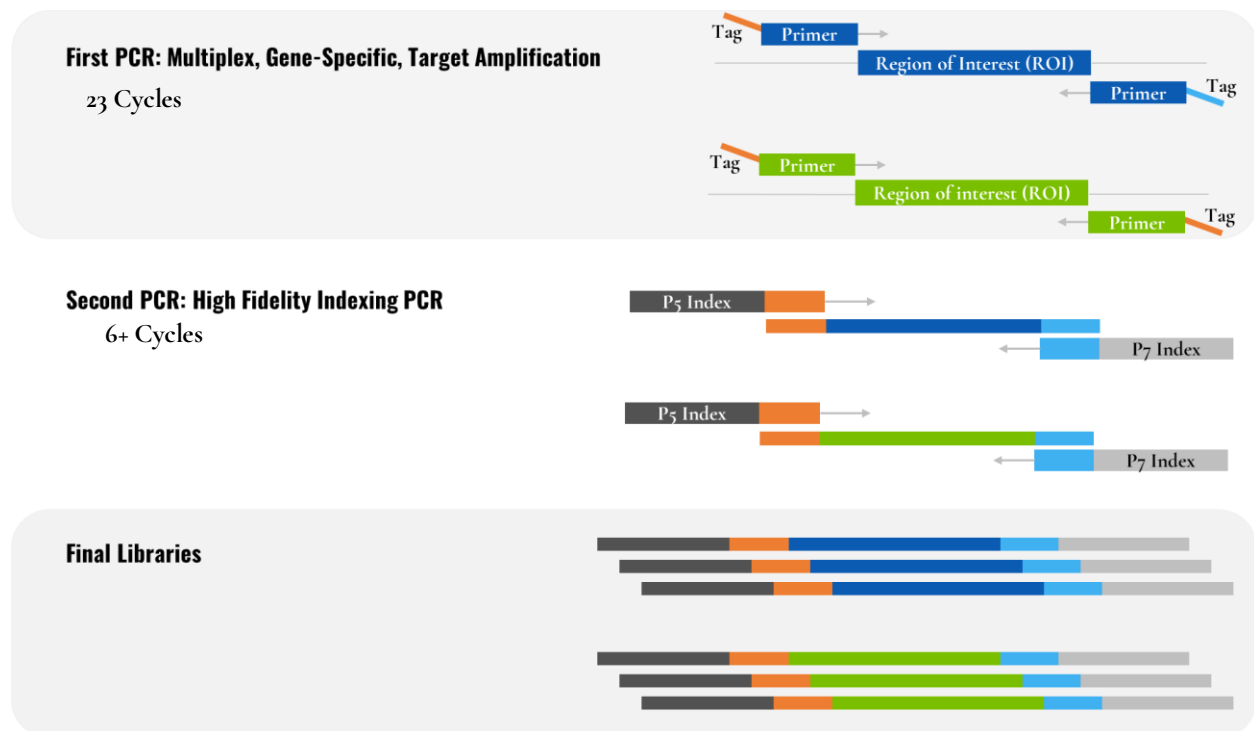


Figure 1. Overview of the inheritReveal™ Thalassemia Panel library preparation.

### 3. General Laboratory Guidelines

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, work areas should be cleaned 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. If a sample tube or non-consumable item has fallen to the floor and remained sealed, the outer surface of the item should be thoroughly cleaned before use using 70% alcohol or freshly-made 10% hypochlorite solution. Gloves should be changed after handling a contaminated item.
- **Aliquot reagents:** Frozen reagents should be aliquot into smaller volumes to reduce freeze/thaw cycles. To reduce the risk of stock contamination it is recommended to aliquot from the stock and work from the aliquots. In case of contamination use of aliquots can also help to determine the source more quickly and easily.
- **Multichannel pipettes:** Multichannel pipettes should be used to maintain consistency and efficiency across numerous samples.
- **Pipette tips:** Tips should be changed between each sample to prevent cross-contamination. Any tips that may have become contaminated due to contact with gloves, the lab bench, tube exteriors, etc., should be discarded.
- **Open containers and lids:** To prevent possible contamination from the air, tubes should be kept closed when not directly in use, and plates, troughs, and similar reservoirs should be covered with seals or lint-free laboratory wipes. Additionally, reaching over open containers should be avoided.

## 4. Reagents & Equipment

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

All reagents should be used in designated pre-PCR or post-PCR areas to prevent amplicon contamination. Each area designated for pre- and post-PCR should have dedicated equipment, reagents, and supplies (including gloves, lab coats, etc.) to prevent contamination.

### 4.1 Kit Components

*inheritReveal™ Thalassemia Panel Part No.: HDA-TH-1001-24*

Reagent	Use	Area Use	Storage
PCR Gene-Specific 2x Master Mix	Gene-Specific PCR	Pre-PCR	-25° to -15°C
Thalassemia v2 Oligo Pool	Gene-Specific PCR	Pre-PCR	-25° to -15°C
PCR Indexing 4x Master Mix	Indexing PCR	Pre-PCR	-25° to -15°C

### 4.2 Indexing Kits

Reagent	Part Number	Use	Area Use	Storage
Pillar Custom Indexing Primers <b>Kit A</b> , indices PI501-8, PI701-4 (32 combinations - 96 reactions)	IDX-PI-1001-96	Indexing PCR	Pre-PCR	-25° to -15°C
Pillar Custom Indexing Primers <b>Kit D</b> , indices PI501-8, PI701-12 (96 combinations - 192 reactions)	IDX-PI-1004-192	Indexing PCR	Pre-PCR	-25° to -15°C

*Only one index kit is needed per assay. Multiple options are available to meet a variety of throughput needs.*

### 4.3 User-Supplied Reagents

Reagent	Area Use	Supplier
10N NaOH or 1N NaOH	Post-PCR	General lab supplier
AMPure XP Beads	Post-PCR	Beckman Coulter, Cat# A63881 or A63880
Ethanol, 200 proof for molecular biology	Post-PCR	General lab supplier
Nuclease-free water	Post-PCR	General lab supplier
Qubit dsDNA High Sensitivity Assay kit	Post-PCR	Invitrogen, Cat# Q32851 or Q32854
Qubit dsDNA Broad Range Assay kit <sup>1</sup>	Post-PCR	Invitrogen, Cat# Q32850 or Q32853
Agarose gel, 2% (optional) <sup>1</sup>	Post-PCR	General lab supplier
DNA molecular weight markers (optional) <sup>1</sup>	Post-PCR	General lab supplier
TapeStation or equivalent <sup>1</sup>	Post-PCR	Agilent Technologies
10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)	Post-PCR	Teknova, Cat# T7724
PhiX Control v3	Post-PCR	Illumina, Cat# FC-110-3001
HT1 Hybridization buffer	Post-PCR	Illumina, Cat# 20015892
200 mM Tris-HCl, pH 7.0 (optional) <sup>2</sup>	Post-PCR	General lab supplier

<sup>1</sup> The Qubit dsDNA High Sensitivity Assay kit is the primary DNA quantitation assay used throughout this protocol. Additional DNA quantification can optionally be performed using the Qubit dsDNA Broad Range Assay kit, an agarose gel, or using TapeStation or equivalent.

<sup>2</sup> The 200 mM Tris-HCl, pH 7.0 reagent is only required for denaturing libraries for sequencing on the Illumina NextSeq or MiniSeq. If sequencing on the MiSeq this reagent is not needed.

## 4.4 Other Consumables

### Compatible Sequencing Reagents

Sequencing Reagent Kit	Supplier	Catalog No.
MiniSeq™ Mid Output kit (300 cycles)	Illumina	FC-420-1004
MiniSeq™ High Output kit (300 cycles)	Illumina	FC-420-1003
MiSeq™ Reagent Nano kit v2 (300 cycles) ‡	Illumina	MS-103-1001
MiSeq™ Reagent Micro kit v2 (300 cycles) ‡	Illumina	MS-103-1002
MiSeq™ Reagent kit v2 (300 cycles) ‡	Illumina	MS-102-2002
MiSeq™ Reagent kit v3 (600 cycles) ‡	Illumina	MS-102-3003
NextSeq™ 500/550 Mid Output v2.5 kit (300 cycles) ‡	Illumina	20024905
NextSeq™ 500/550 High Output v2.5 kit (300 cycles)	Illumina	20024908
NextSeq™ 1000/2000 P1 Reagents (300 cycles)	Illumina	20050264
NextSeq™ 1000/2000 P1 Reagents (600 cycles)	Illumina	20075294
NextSeq™ 1000/2000 P2 Reagents v3 (300 cycles)	Illumina	20046813
NextSeq™ 1000/2000 P2 300M Reagents (600 cycles)	Illumina	20075295
NextSeq™ 2000 P3 Reagents (300 cycles)	Illumina	20040561

‡ Indicates the flowcells the inheritReveal™ Thalassemia Panel has been validated on. However, libraries generated using this protocol are compatible with all Illumina sequencers.

### General Laboratory Consumables

In addition to the consumables listed below other general laboratory supplies needed to carry out the protocol include gloves, benchtop cooler, tube racks, etc.

Consumable	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	Corning Life Sciences, Cat# 6551 or equivalent
Microplate sealing film	Pre- and Post-PCR	Corning Life Sciences, Cat# 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 Scientific, Cat# 13-681-506 or equivalent
Qubit Assay tubes	Post-PCR	Invitrogen, Cat# Q32856

## 4.5 Equipment

Equipment	Area Use	Supplier
Centrifuge adapted for PCR plates, tabletop	Pre- and Post-PCR	General lab supplier
Gel electrophoresis apparatus (optional)*	Post-PCR	General lab supplier
TapeStation or equivalent	Post-PCR	Agilent Technologies
Magnetic stand for 96 wells	Post-PCR	Life Technologies, Cat# 12331D or 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, Cat# Q33216/Q33218
Vortex mixer	Pre- and Post-PCR	General lab supplier

\* The Qubit dsDNA High Sensitivity kit is the primary DNA quantitation assay used throughout this protocol. Additional DNA quantification can optionally be performed using either an agarose gel or the TapeStation or equivalent.

## 5. Workflow

The following chart demonstrates the workflow for performing the inheritReveal™ Thalassemia Panel library preparation.

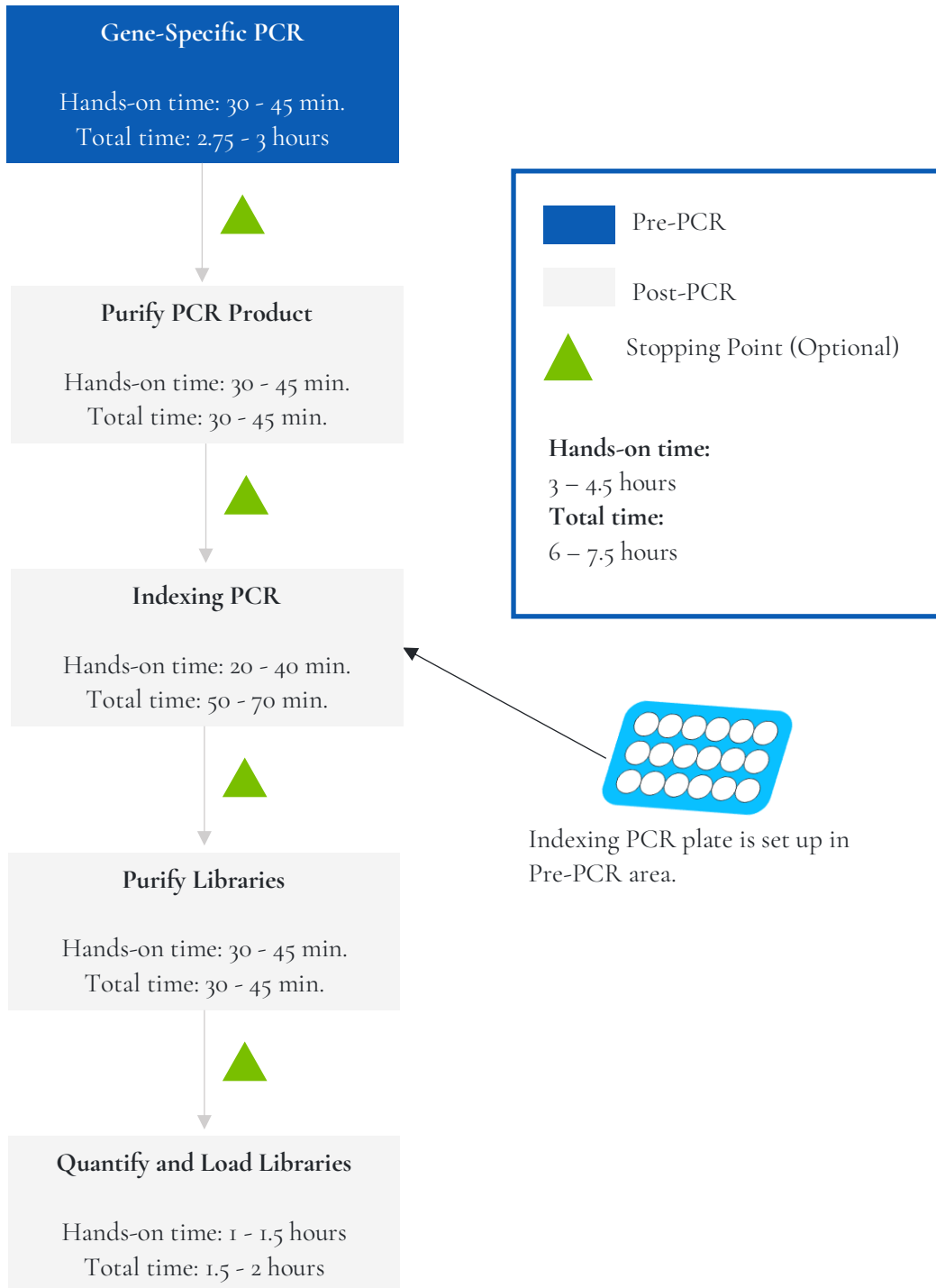


Figure 2. Library preparation workflow for inheritReveal™ Thalassemia Panel. The workflow can be completed within one day but contains multiple optional stopping points for users with time constraints.

## 6. DNA Input Information

### Inclusion of Normal Samples

The Thalassemia analysis is based on a 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, 3 – 5 (**minimum 2**) in-run normal (negative) samples should be provided. It is best if the negative control sample is derived from the same tissue type, handled with the same procedure, and prepared using the same DNA extraction method as the patient samples. If negative controls are not provided, SNV and indel variant calls will still be generated, but CNV calls will not be generated.

### DNA Input Recommendations

The following protocol includes information for preparing libraries using genomic DNA from white blood cells.

The recommended DNA input per reaction:

- Genomic DNA: 10 – 75 ng
- For best performance, normalize the DNAs and the normal negative control DNAs to the same input amount and start with 20 – 30 ng of DNA per reaction. At 30 ng DNA input and above, CNV detection is robust within 30% input range variations (e.g., 30 ng +/- 10 ng).

Symbol	Description
	<b>Information:</b> Information that follows this symbol is important and may require action.
	<b>Optional Stopping Point:</b> A point in the workflow at which work may be safely paused, and samples can be stored appropriately.
	<b>Caution:</b> Information that follows this symbol is critical to the workflow. Information following this symbol should not be skipped or ignored.

*Symbols used throughout this protocol and their associated meaning.*

## 7. Library Preparation Protocol

<b>Hands-on Time</b>	3 – 4.5 hours
<b>Total Time</b>	6 – 7.5 hours

### 7.1 Gene-Specific PCR (GS-PCR) & Purification

<b>Hands-on Time</b>	30 – 40 min.
<b>Total Time</b>	2.75 – 3 hours

#### Amplify Genomic DNA Targets

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

For this portion of the protocol prepare an ice bucket and keep the gene-specific PCR Master Mix (GS-PCR MMX) and the custom oligo pool on ice.

See recommended DNA input quantities in the section titled “DNA Input Information.”

- Prepare Gene-Specific PCR Mix 1:** Vortex and centrifuge the GS-PCR MMX reagent and the oligo pool before use. For each PCR reaction, the volume of each component is listed below.



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

GS-PCR Mix 1	
Reagent	Volume (μL)
PCR Gene-Specific 2x Master Mix	12.5
Thalassemia v2 Oligo Pool	5.0
<b>Subtotal</b>	<b>17.5</b>

- Transfer:** Transfer 17.5 μL of GS-PCR Mix 1 to each sample well in a PCR plate, strip tube, or PCR tube.
- Dilute input DNA:** Add 7.5 μL of DNA (diluted if necessary) to each sample well containing GS-PCR Mix 1. Add 7.5 μL of nuclease-free water to the no-template control well.

Final GS-PCR Mix	
Reagent	Volume (μL)
GS-PCR Mix 1	17.5
Diluted DNA (or water)	7.5
<b>Total</b>	<b>25.0</b>

\* The DNA concentration can be determined using the Qubit dsDNA HS Assay Kit or the Qubit dsDNA BR Assay Kit.

- Seal and mix:** Carefully seal the reactions and vortex for 10 – 15 seconds.

5. **Spin:** Briefly centrifuge the reactions to remove any air bubbles from the bottom of the wells and to spin down droplets from the seal or side walls.
6. **Perform GS-PCR:** Perform the following program with the heated lid on:

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



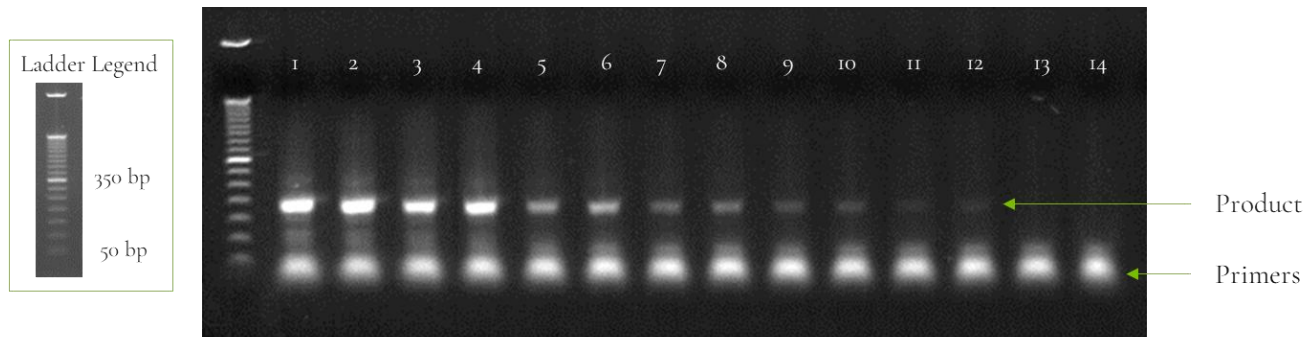
Do not leave reactions on the thermocycler overnight at 8°C. Once cycling is complete the reactions can be stored at -20°C.



**Optional Stopping Point:** The gene-specific PCR reactions may be stored at -20°C after cycling is complete.

### Gel Image After Gene-Specific PCR

The following image is an example of samples after gene-specific PCR 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).

*Figure 3. Analysis of GS-PCR material using various inputs on 2% agarose gel.*

## Purify the Gene-Specific PCR Product

<b>Hands-on Time</b>	30 – 45 min.
<b>Total Time</b>	30 – 45 min.

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

Before beginning the purification:

- Remove AMPure XP beads from 4°C and incubate at room temperature for at least 30 minutes before use.



**Caution:** Ensure that the AMPure bead solution reaches room temperature before performing the purification. The temperature of the bead solution can have adverse effects on the purification process.

- If gene-specific PCR products were stored at -20°C, allow to thaw at room temperature and then proceed immediately to purification.
- Prepare 70% ethanol by mixing three parts of water with seven parts of absolute ethanol in a conical tube, invert tube a few times to mix and dispense sufficient volume to a disposable trough for convenient dispensing using a multichannel pipette.



**Caution:** Fresh 70% ethanol is required for optimal results.

### Gene-Specific Product Purification


- If condensation has formed or if reactions were stored at -20°C briefly centrifuge the reactions upon thawing. Carefully remove the seal.
- Mix Beads:** Vortex AMPure XP beads thoroughly until all beads are well-dispersed.




**Caution:** Ensure that the AMPure bead solution is homogenous. A non-uniform distribution can have unpredictable effects on the purification process.

- Add water to sample:** Add 25 µL of nuclease-free water to each well containing GS-PCR product to bring the reaction volume to 50 µL.
- Add beads:** Add 60 µL of AMPure beads (equivalent to a 1.2x bead ratio) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge the samples and mix again.
- Bind GS-PCR product to beads:** Incubate for 5 minutes at room temperature.
- Separate beads containing GS-PCR product:** Place the PCR plate on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
- Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.

8. **Wash beads:** With the PCR plate still on the magnetic rack add **150 µL** of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate at room temperature for 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 the indexing PCR is completed.
10. **Remove remaining ethanol wash:** Centrifuge for 10–15 seconds, place the PCR plate back on the magnetic rack, and use a 10 or 20 µL tip to remove any trace amounts of ethanol from each well.
11. **Dry beads:** Keep the samples on the magnetic rack and let the beads air dry at room temperature for 2 – 5 minutes.

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

12. **Resuspend beads:** Remove the samples from the magnetic rack and resuspend the dried beads in each well using **32 µL** of nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.

*TIP: After resuspending the beads, cover the reactions and prepare for the indexing PCR step using the Indexing PCR Master Mix. The Indexing PCR Master Mix should be handled in the pre-PCR area. Alternatively, the purified gene-specific PCR product may be stored at -20°C after elution.*

13. **Incubate and Elute:** Incubate at room temperature for 5 minutes.
14. **Separate GS-PCR product from beads:** Place the reactions on a magnetic rack at room temperature until the solution appears clear. Transfer **30 µL** of clear supernatant from each well to a new PCR plate.

 **Optional Stopping Point:** The purified GS-PCR products can be stored at -20°C.

## 7.2 Index PCR & Purification

### Indexing PCR:

<b>Hands-on Time</b>	20 – 40 min.
<b>Total Time</b>	50 – 70 min.

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

For this portion of the protocol prepare a prechilled cooler. The Indexing PCR Master Mix should be kept in the cooler until it is needed.

- Prepare Indexing Primer Mix:** In a new PCR plate add **4 µL** each of the appropriate forward and reverse indexing primers to each sample well that will be used.

Indexing Primer Mix (per reaction)	
Reagent	Volume (µL)
Pi700 Pillar Index	4.0
Pi500 Pillar Index	4.0
<b>Subtotal</b>	<b>8.0</b>

- Prepare Indexing PCR Mix 1:** Briefly vortex and centrifuge the Indexing PCR Master Mix before use. To prepare the Indexing PCR Mix 1 combine the Indexing PCR Master Mix and water with sufficient overage.

Indexing PCR Mix 1 (per reaction)	
Reagent	Volume (µL)
Indexing PCR Master Mix (2x)	25.0
Nuclease-free water	11.0
<b>Subtotal</b>	<b>36.0</b>

- Add Indexing PCR Mix 1 to Indexing Primer Mix:** Transfer **36 µL** of the Indexing PCR Mix 1 to each sample well of the PCR plate in step 1 that contains Indexing Primer Mix. To prevent cross-contamination of indices, be sure to change tips between each well.

Indexing PCR Mix 2 (per reaction)	
Reagent	Volume (µL)
Indexing Primer Mix	8.0
Indexing PCR Mix 1	36.0
<b>Subtotal</b>	<b>44.0</b>

The following steps should be performed in a post-PCR area. Cover or seal the reactions before transferring from the pre-PCR area to the post-PCR area.

If the purified GS-PCR products were stored at -20°C after the GS-PCR purification, ensure that they have been thawed at room temperature thoroughly before proceeding.

4. **Add purified GS-PCR product:** Add 6  $\mu\text{L}$  of purified GS-PCR product into the appropriate wells containing Indexing PCR Mix 2.

Index PCR Final Mix (per reaction)	
Reagent	Volume ( $\mu\text{L}$ )
Indexing PCR Mix 2	44.0
Purified Gene-Specific PCR product	6.0
<b>Total</b>	<b>50.0</b>

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

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

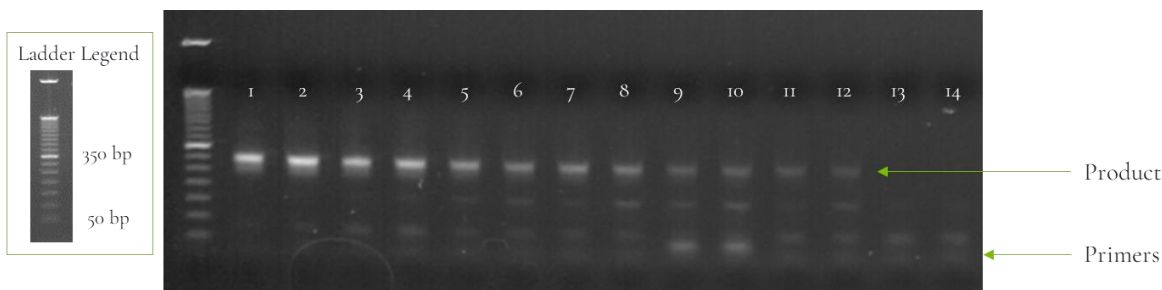
\* Additional Indexing PCR cycles can be performed if final library yield is low or initial DNA input is below recommended minimum.



**Optional Stopping Point:** The indexed libraries can be stored at -20°C after cycling is complete.

### Gel Image After Indexing PCR

The following image is an example of samples after indexing PCR 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).

Figure 4. Analysis of indexing PCR material prior to purification, using various inputs on 2% agarose gel.

## Indexing PCR: Purify the Libraries

<b>Hands-on Time</b>	30 – 45 min.
<b>Total Time</b>	30 – 45 min.

Before beginning the purification:

- Keep AMPure XP beads at room temperature while the Indexing PCR is being performed unless reactions will be stored at -20°C after the program is complete.



**Caution:** Ensure that the AMPure bead solution reaches room temperature before performing the purification. The temperature of the bead solution can have adverse effects on the purification process.

- If the indexed libraries were stored at -20°C remove them from the freezer to thaw thoroughly to ambient temperature before purification. After samples have thawed, briefly centrifuge to remove any droplets from the side walls.

Library Purification

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

1. **Mix beads:** Vortex AMPure XP beads thoroughly until all beads are well dispersed.



**Caution:** Ensure that the AMPure bead solution is homogenous. A non-uniform distribution can have unpredictable effects on the purification process.

2. **Add beads:** Add 50 µL beads (equivalent to a 1.0x bead ratio) to each well. Pipette the mixture up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.
3. **Bind libraries to beads:** Incubate for 5 minutes at room temperature.
4. **Separate libraries on beads:** Place the PCR plate on a magnetic rack until the solution appears clear, which can take up to 5 minutes.
5. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.
6. **Wash beads:** With the PCR plate still on the magnetic rack add 150 µL of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove the supernatant from each well.



**Caution:** 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.

7. **Second wash:** Repeat step 6 for a second 70% ethanol wash. Remove the supernatant from each well.

8. **Remove remaining ethanol wash:** Centrifuge for 10 – 15 seconds, place the PCR plate back on the magnetic rack, and use a 10 or 20  $\mu\text{L}$  tip to remove any trace amounts of ethanol from each well.

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



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

10. **Resuspend beads:** Remove the PCR plate from the magnetic rack and resuspend the dried beads in each well using 32  $\mu\text{L}$  of nuclease-free water. Gently pipette the bead suspension up and down 10 times. If bubbles form on the bottom of the wells briefly centrifuge and mix again.

11. **Elute libraries:** Incubate at room temperature for 5 minutes.

12. **Separate libraries from beads:** Place the bead suspensions on the magnetic rack at room temperature until the solution appears clear. Transfer 30  $\mu\text{L}$  of clear supernatant to a new plate.

*TIP: During the incubation and magnetic separation of the beads, cover the samples with microplate sealing film and prepare the solutions needed for quantitation performed in the next section.*

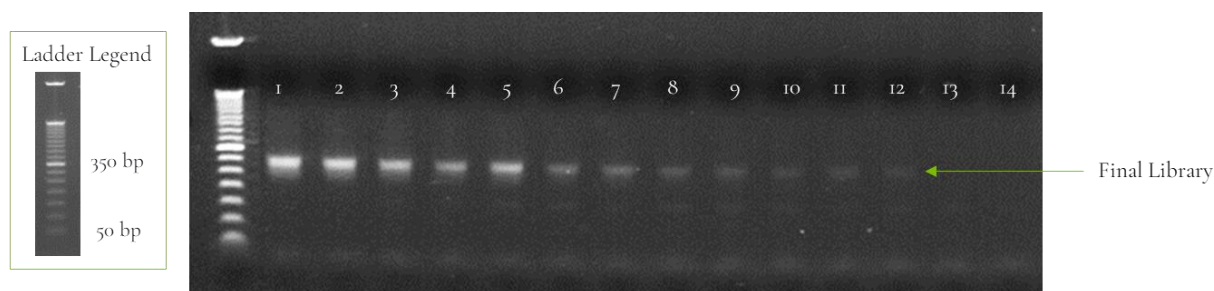
13. **Quantification:** Analyze an aliquot of each library per the instructions in the next section.



**Optional Stopping Point:** The purified libraries can be stored at 4°C for up to 3 days, or at -20°C for longer-term storage.

## Final Library Gel Image

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

*Figure 5. Gel analysis of final library material.*

### 7.3 Quantitation and Normalization of Purified Libraries

Prior to sequencing, libraries must be quantified, normalized, and then pooled together. The following section describes how to quantify libraries using the Qubit system. Other library quantification methods, such as qPCR quantification kits or Bioanalyzer, may be used according to the manufacturer's protocol.

#### Qubit Quantitation

<b>Hands-on Time</b>	30 – 45 min.
<b>Total Time</b>	30 – 45 min.

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 the Qubit working solution.

*For example, 2000  $\mu\text{L}$  is sufficient buffer for 10 readings (8 samples + 2 standards). Combine 1990  $\mu\text{L}$  of Qubit dsDNA HS buffer and 10  $\mu\text{L}$  HS reagent.*



**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  $\mu\text{L}$  of Qubit working solution into two tubes for standard 1 and standard 2, and then add 10  $\mu\text{L}$  of each standard to the corresponding tube.



**Caution:** New standard dilutions should be prepared with the libraries to be quantified. Do not re-use standard dilutions from previous experiments.

4. **Prepare samples:** Transfer 198  $\mu\text{L}$  of Qubit working solution to each 0.5 mL tube, and then add 2  $\mu\text{L}$  of each library to its corresponding Qubit tube (1:100 dilution).
5. **Mix and spin:** Vortex to mix and then centrifuge the tubes briefly.
6. Incubate the tubes at room temperature for 2 minutes.
7. **Measure concentration:** Measure the concentration of each library on the Qubit 2.0 Fluorometer per the manufacturer's instructions. Use the dsDNA High Sensitivity assay to read standards 1 and 2 followed by the libraries.

If any concentration is above the linear range of the instrument, prepare a new dilution by combining 199  $\mu\text{L}$  Qubit working solution and 1  $\mu\text{L}$  library (1:200 dilution). Repeat steps 5–7.

8. **Calculate concentration:** 1 ng/ $\mu\text{L}$  of library is equal to 5 nM. Example calculation is below. Adjust dilution factor accordingly.

2  $\mu\text{L}$  of library + 198  $\mu\text{L}$  Qubit working solution:

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



**Optional Stopping Point:** Once libraries have been quantified either proceed with normalization and pooling or else keep them stored at 4°C for up to 3 days. Store libraries at -20°C for long-term storage.

## Normalization and Pooling

<b>Hands-on Time</b>	30 – 45 min.
<b>Total Time</b>	30 – 45 min.

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



If sequencing on the MiniSeq, libraries should be normalized to **1 nM** prior to pooling. For all other Illumina sequencing platforms, libraries should be normalized to **5 nM** prior to pooling.



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

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

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

2. **Mix and spin:** Mix the 5 nM libraries thoroughly by vortexing and then centrifuge briefly.
3. **Prepare library pool:** Label a new 1.5 mL microtube for the library pool. Prepare an equimolar 5 nM mixture of libraries by combining each library at equal volume (e.g., mixing 5 µL of each 5 nM library). Gently pipette the entire solution up and down 10 times to mix thoroughly. Quickly vortex the pool and then briefly centrifuge.
4. **Quantify library pool (recommended):** It is recommended that the library pool be quantified using Qubit or another library quantitation method (qPCR) to ensure the pool is at 5 nM ( $\pm 10\%$ ) to prevent poor sequencing performance. If the final dilution is not 5 nM ( $\pm 10\%$ ), adjust the dilution for loading the sequencer accordingly to obtain the desired concentration.



**Optional Stopping Point:** The normalized libraries can be stored at 4°C overnight for sequencing the next day. For longer storage, the normalized libraries can be stored at -20°C.

## 8. Sequencing

### Prepare Diluted Libraries for Sequencing

<b>Hands-on Time</b>	30 – 40 min.
<b>Total Time</b>	30 – 40 min.

The libraries generated using this protocol can be multiplexed and sequenced on Illumina® sequencers. Table 1 outlines the sequencing parameters and the recommended per-sample sequencing coverage.

The number of samples that can be multiplexed together is dependent on several factors, among them are the estimated throughput of the flowcell and sequencing platform, the desired sequencing depth, as well as the number of unique index combinations available.

The estimated **maximum** number of samples that can be multiplexed on a single flowcell using each kit is displayed in Table 2. Select the appropriate sequencing kit based on the number of samples to be sequenced.

Sequencing Configuration	Index	Recommended Coverage	Recommended Reads Per Sample
2 x 150	<b>Dual</b> (8 bp each)	Mean: 1000X Mean: 200X	>262,000 PE reads

Table 1. Sequencing recommendations.

Sequencing Flowcell	Estimated Instrument Output (million PE reads)	Estimated Maximum Sequencing Batch Size
<b>MiSeq Nano</b>	2	6
<b>MiSeq Micro</b>	8	27
<b>MiSeq v2</b>	30	103
<b>MiSeq v3</b>	50	171
<b>NextSeq 500/550 Mid Output</b>	260	>800

Table 2. Multiplexing recommendations for Illumina sequencers. Calculations assume ≥ 90% effective on-target rate after read mapping and minimum segment coverage > 20% of the mean coverage.

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

For this portion of the protocol prepare a prechilled cooler.

### Sequencing Using Illumina MiSeq™ v2 Reagents

**MiSeq v2 recommended final library pool loading concentration: 10 pM**

**MiSeq v2 recommended PhiX spike-in concentration: 12.5 pM**

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

1. **Normalize:** Dilute libraries to 5 nM, as demonstrated in the previous section "Quantification and Normalization of Purified Libraries."
2. **Prepare 0.2 N NaOH:** Label a new 1.5 mL microtube for 0.2 N NaOH. Prepare the NaOH by combining **800 µL** of nuclease-free water with **200 µL** of 1 N NaOH. Vortex the solution to mix.
 

*NOTE: Alternatively, prepare a 1 N NaOH solution by combining 500 µL of 10 N NaOH with 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.*
3. **Denature the library pool:** Label a new 1.5 mL microtube for the denatured 25 pM library pool.
  - a. Denature the 5 nM library pool by combining **5 µL** of the library pool and **5 µL** of the freshly prepared 0.2 N NaOH.
  - b. Vortex thoroughly for 10 seconds and centrifuge for 1 minute.
  - c. Let the solution stand at room temperature for 5 minutes.
  - d. Add **990 µL** of Illumina's HT1 hybridization buffer to the denatured library pool.
  - e. Invert the mixture several times, centrifuge briefly, and place on ice.
4. **Dilute library pool to 10 pM:** Label a new 1.5 mL microtube for the 10 pM library mix. Combine **240 µL** of the 25 pM library pool with **360 µL** of Illumina's HT1 hybridization buffer. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, centrifuge briefly, and place on ice.
5. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded onto the sequencing flowcell. Combine **594 µL** of the 10 pM library mix with **6 µL** of 12.5 pM PhiX library control. Briefly vortex, centrifuge, and place on ice.
6. **Load MiSeq cartridge:** Using a clean 1000 µL tip puncture the foil cap above the sample loading well on the MiSeq cartridge. Load the **600 µL** library pool and PhiX mixture into the cartridge, ensuring that the solution has reached the bottom of the well.
7. **Run the MiSeq:** Sequence 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. See "MiSeq System User Guide" (part #15027617).
8. **Store** diluted libraries and mixtures at -20°C for long-term storage.

### Sequencing Using Illumina MiSeq™ v3 Reagents

**MiSeq v3 recommended final library pool loading concentration: 12.5 pM**

**MiSeq v3 recommended PhiX spike-in concentration: 12.5 pM**

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

1. **Normalize:** Dilute libraries to 5 nM, as demonstrated in the previous section "Quantification and Normalization of Purified Libraries."
2. **Prepare 0.2 N NaOH:** Label a new 1.5 mL microtube for 0.2 N NaOH. Prepare the NaOH by combining **800 µL** of nuclease-free water with **200 µL** of 1 N NaOH. Vortex the solution to mix.  
  
*NOTE: Alternatively, prepare a 1 N NaOH solution by combining 500 µL of 10 N NaOH with 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.*
3. **Denature the library pool:** Label a new 1.5 mL microtube for the denatured 25 pM library pool.
  - a. Denature the 5 nM library pool by combining **5 µL** of the library pool and **5 µL** of the freshly prepared 0.2 N NaOH.
  - b. Vortex thoroughly for 10 seconds and centrifuge for 1 minute.
  - c. Let the solution stand at room temperature for 5 minutes.
  - d. Add **990 µL** of Illumina's HT1 hybridization buffer to the denatured library pool.
  - e. Invert the mixture several times, centrifuge briefly, and place on ice.
4. **Dilute library pool to 12.5 pM:** Label a new 1.5 mL microtube for the 12.5 pM library mix. Combine **300 µL** of the 25 pM library pool with **300 µL** of Illumina's HT1 hybridization buffer. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, centrifuge briefly, and place on ice.
5. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded onto the sequencing flowcell. Combine **594 µL** of the 10 pM library mix with **6 µL** of 12.5 pM PhiX library control. Briefly vortex, centrifuge, and place on ice.
6. **Load MiSeq cartridge:** Using a clean 1000 µL tip puncture the foil cap above the sample loading well on the MiSeq cartridge. Load the **600 µL** library pool and PhiX mixture into the cartridge, ensuring that the solution has reached the bottom of the well.
7. **Run the MiSeq:** Sequence 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. See "MiSeq System User Guide" (part #15027617).
8. **Store** diluted libraries and mixtures at -20°C for long-term storage.

## Sequencing Using Illumina's NextSeq™ 500/550 Reagents

**Mid Output recommended final library pool loading concentration: 1.8 pM**

**Mid Output recommended PhiX spike-in concentration: 1.8 pM**

- 1. Normalize:** Dilute libraries to 5 nM, as demonstrated in the previous section “*Normalization and Pooling*.”
- 2. 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.  
  
*NOTE: Alternatively, prepare a 1 N NaOH solution by combining 500 µL of 10 N NaOH with 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.*
- 3. Denature the library pool:** Label a new 1.5 mL microtube for the denatured 25 pM library pool.
  - Denature the 5 nM library pool by combining **5 µL** of the library pool and **5 µL** of the freshly prepared 0.2 N NaOH.
  - Vortex thoroughly for 10 seconds and centrifuge for 1 minute.
  - Let the solution stand at room temperature for 5 minutes.
  - Add **5 µL** of 200 mM Tris-HCl, pH 7.0.
  - Vortex briefly and centrifuge for 1 minute.
  - Add **985 µL** of Illumina's HT1 hybridization buffer to the denatured library mix.
  - Vortex briefly and centrifuge for 1 minute.
- 4. Dilute 25 pM library pool to 1.8 pM:** Dilute the denatured library to 1.8 pM in 1400 µL by combining **101 µL** of the 25 pM denatured library mix with **1299 µL** of Illumina's HT1 hybridization buffer. Invert to mix and centrifuge briefly.
- 5. 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 with **13 µL** of a 1.8 pM PhiX library control. Briefly vortex, centrifuge, and place on ice.
- 6. 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 into the cartridge and ensure the solution has reached the bottom of the cartridge well.
- 7. Run the NextSeq:** Sequence 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. See “NextSeq System User Guide” (part #15046563 or 15069765).
- 8. Store** diluted libraries and mixtures at -20°C for long-term storage.

## Preparing a Sample Sheet for Sequencing

*TIP: 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 Appendix A. For the i5 indexing primers, indexing on the NextSeq requires the reverse complement of the barcode sequence. The correct barcode sequences for sequencing on the MiSeq and the NextSeq are 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 the index combination 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 the 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”.

## 9. Troubleshooting Library Preparation

### Low Yield of Gene-Specific Product

Potential Cause	Solution
DNA quantity or quality	The recommended input for the assay is 10 – 75 ng of genomic DNA, and a minimum of 20 ng if the genomic DNA is degraded. 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

Potential Cause	Solution
Improper AMPure purification	Incomplete AMPure purification or loss of gene-specific product will affect the indexing PCR reaction. The purified gene-specific product can be checked on an agarose gel to ensure the product was not lost, and that the clean-up was sufficient to remove excess primers.
	The AMPure bead ratio and ethanol concentration affect the PCR clean-up. Ensure that the correct AMPure bead concentration was used for clean-up, and fresh 70% ethanol was used for the wash.
	Leftover ethanol from the wash steps can hinder the PCR reaction. Remove as much of the ethanol during the final wash step with a pipette and dry the beads to ensure the residual ethanol has evaporated.

### Low Yield of Final Library

Potential Cause	Solution
DNA quantity or quality	The recommended input for the assay is 10 – 75 ng of genomic DNA, and a minimum of 20 ng if the genomic DNA is degraded. Higher quantities may be necessary for low- or poor-quality samples.
	Run the product from the gene-specific PCR on an agarose gel to check the yield.

<b>Improper AMPure purification</b>	Incomplete AMPure purification or loss of product will affect the final yield. The purified product can be checked on an agarose gel to ensure that the product was not lost during PCR clean-up.
	The AMPure bead ratio and ethanol concentration affect the PCR clean-up. Ensure that the correct AMPure bead concentration was used for clean-up, and fresh 70% ethanol was used for the wash.

### Amplicon Contamination in No-Template Control

Potential Cause	Solution
<b>Cross-contamination</b>	Make sure to change tips between samples and avoid waving tips over tubes or plates.
	Poor sealing or residual liquid in tips can cause contamination of nearby samples. If possible, leave adjacent wells empty between samples.
	Workspaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination.
	Periodically clean the workspace, floor, equipment, and instrumentation with a laboratory cleaning solution to break down amplicons on surfaces.  Recommended cleaning solutions are 10% bleach, 70% isopropanol, or 70% ethanol.

### Sequencing Performance

Potential Cause	Solution
<b>Improper normalization and pooling of libraries</b>	Confirm that the appropriate loading concentration was used for the applicable sequencing instrument.
	Check the 5 nM (or 1 nM if sequencing on the MiniSeq) library mix using Qubit or RT-PCR. Dilute the denatured library mix as needed to adjust for the difference in concentration.
<b>Improper library quantitation</b>	Improper library quantitation may result in artificially high or low yields, which affects downstream normalization.  Re-quantitate the final libraries and/or the normalized libraries to check for the expected values.

<b>Improper AMPure purification</b>	<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 final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.</p>
	<p>The AMPure bead ratio and ethanol concentration affect the PCR clean-up. Ensure that the correct AMPure bead concentration was used for clean-up, and fresh 70% ethanol was used for the wash.</p>

## 10. Appendix A: Index Sequences

Indexing primers highlighted in yellow have the same barcode sequences as Illumina TruSeq Custom Amplicon (TSCA) indices.

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

i5 Index Sequences		
Index ID	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

## II. Appendix B: Thalassemia Analysis using PiVAT®

See the *PiVAT User Manual (Doc. No. UM-0073)* for detailed instructions on initializing the analysis & understanding the output in PiVAT. Refer to Appendix D for Thalassemia CNV Analysis.