# ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel

## Library Preparation User Guide

FOR RESEARCH USE ONLY

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Pillar Biosciences
pillar-biosciences.com
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## INTRODUCTION

The ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel utilizes our proprietary SLIMamp® (stem-loop inhibition mediated amplification) technology, allowing researchers to amplify regions of interest in a simple, single tube, multiplex reaction. Subsequent libraries are designed for sequencing on the Illumina platform using a paired-end read length of 150 (2x150). The ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel contains 283 amplicons and numerous gene regions of interest for researchers looking to explore the genetic sequences of both germline and formalin-fixed paraffin-embedded (FFPE) DNA from breast cancer samples.

The work flow of the ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel can be performed and loaded onto the sequencing instrument by researchers within one day. The protocol also contains numerous stopping points for users who have time limitations.

### How Does the ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel Work?

A pair of DNA oligos designed for each region of interest, or hot spot, are used in the first round of gene-specific PCR (GS-PCR) and the products are subsequently purified via size selection. After purification, a second round of PCR adds index adaptors and P5 and P7 sequences to each library for sample tracking and sequencing on Illumina's flow cells. Those products are further purified and sequenced (Figure 1).

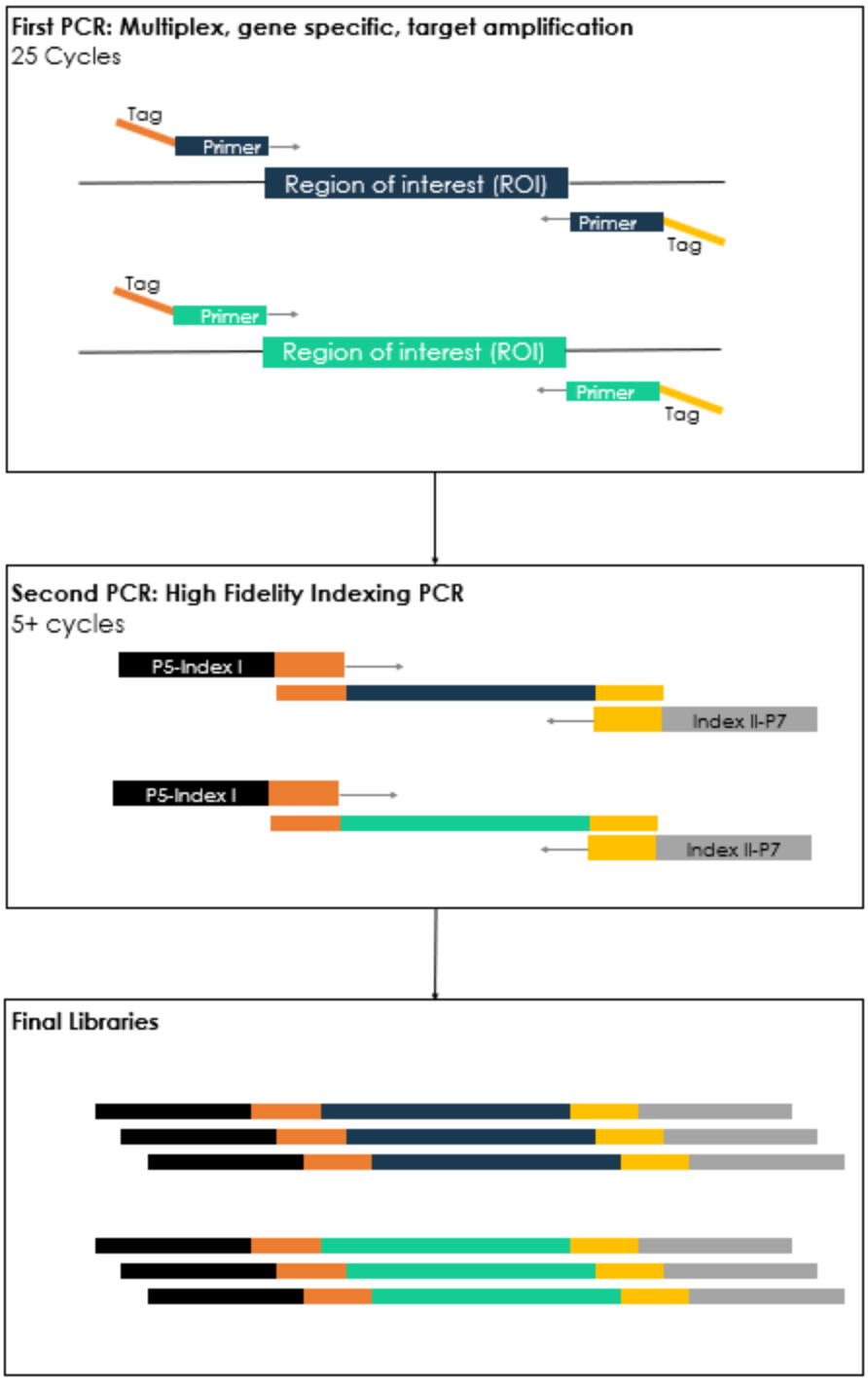


**Figure 1.** Overview of the ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel library preparation.

**REVISION HISTORY**

2019-12: Creation of version 0.1.0
2019-12: Edited GS-PCR purification step in version 0.1.1
2020-07: Added Sample Set-Up and CNV analysis instructions in version 0.2.0

## GETTING STARTED

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

### Components of the ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel

| Reagent | Use | Area Use | Storage |
|---|---|---|---|
| *Gene-Specific PCR Master Mix (2x)* | Gene-specific PCR | Pre-PCR | -15° to -25°C |
| *BR283 oligo pool* | Gene-specific PCR | Pre-PCR | -15° to -25°C |
| *Exonuclease I* | Gene-specific PCR | Post-PCR | -15° to -25°C |
| *Indexing PCR Master Mix (2x)* | Indexing PCR | Pre-PCR | -15° to -25°C |

### ONCO/Reveal ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV 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.

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

PCR should have dedicated equipment, supplies, and reagents to prevent contamination.

**User-supplied Reagents**

| Reagent | Area use | Supplier |
|---|---|---|
| *10 N NaOH or 1 N NaOH* | Post-PCR | General lab supplier |
| *Agencourt AMPure XP Beads* | Post-PCR | Beckman Coulter, #A63881/ #A63880 |
| *Ethanol, 200 proof for molecular biology* | Post-PCR | General lab supplier |
| *Nuclease-free water* | Pre- and Post-PCR | General lab supplier |
| *Qubit dsDNA High Sensitivity assay kit* | Post-PCR | Invitrogen, #Q32851/ #Q32854 |
| *Agarose gel, 2% (optional)* | Post-PCR | General lab supplier |
| *DNA molecular weight markers (optional)* | Post-PCR | General lab supplier |
| ***Or** Bioanalyzer High Sensitivity DNA Analysis (optional)* | Post-PCR | Agilent #5067-4627/ #5067-4626 |
| *Uracil-DNA glycosylase (UDG) (optional)* | Pre-PCR | NEB, #M0280S or #M0280L |
| *10 mM Tris-HCl w/ 0.1% Tween-20, pH 8.5 (optional)* | Post-PCR | Teknova, Cat#T7724 |

**Compatible Illumina Reagent Kits**

| | |
|---|---|
| *MiSeq reagent 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, #6551 or equivalent |
| *Microplate sealing film* | Pre- and post-PCR | Axygen, #PCR-TS or equivalent |
| *Conical tubes, 15 mL* | Pre- and post-PCR | General lab supplier |
| *Conical tubes, 50 mL* | Post-PCR | General lab supplier |
| *Low retention, aerosol filter pipette tips* | Pre- and post-PCR | General lab supplier |
| *Solution basin (trough or reservoir)* | Pre- and post-PCR | Fisher, #13-681-506 or equivalent |
| *Qubit Assay tubes* | Post-PCR | Invitrogen, #Q32856 |

## Equipment Requirements

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

Other general lab supplies needed to carry out the protocol include laboratory gloves, ice, ice buckets, tube racks, etc.
For reagents, consumables, and equipment required in both pre- and post-PCR processes, dedicated supplies (including gloves, lab coats, etc.) should be in both areas.

**BEST PRACTICES**

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

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

**ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel Workflow**

The following chart (Figure 2) demonstrates the workflow for performing the ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel Panel library preparation.

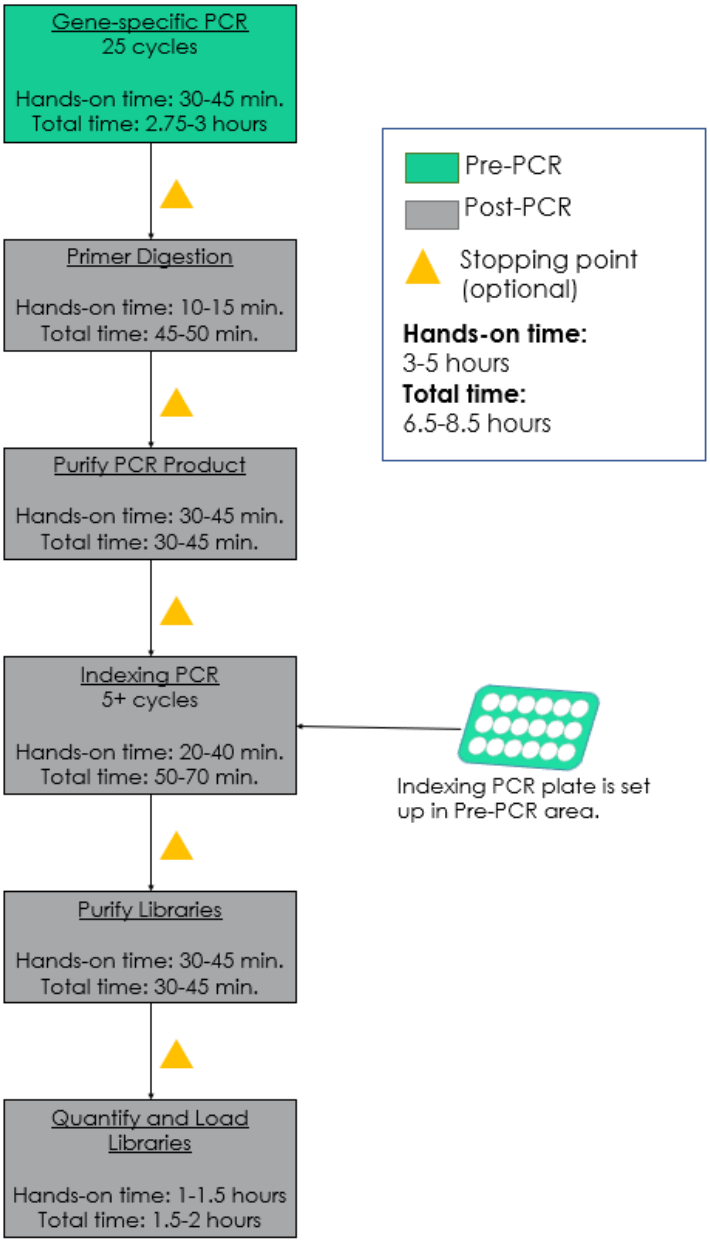


**Figure 2.** The ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel workflow can be completed within a day but contains multiple stopping points for users with time constraints.

**Sample Set-Up**

The CNV analysis is based on double normalization method, including one per-sample normalization and one per-amplicon normalization. The CNV is called by amplicon clusters and exon ID can be identified from the amplicon ID. The cluster CNV call is split by genes and each gene is displayed as one row in the report. All 283 amplicons are used in the CNV analysis.

In order to achieve correct normalization, it is recommended that the user provide 3 to 5 (minimum 2) normal reference samples. Ideally, the normal reference samples will have similar sample condition and preparation process as the CNV positive samples.

Alternatively, if no normal reference sample is provided, BRCA-CNV analysis will be run with the center percentile normalization algorithm. This will be automatically detected by PiVAT. No user parameter adjustment is needed. In this setting, the required minimum number of samples is 10. In addition, the number of samples with the same type of CNV cannot exceed 30% of all samples. It is recommended that the user pool final libraries of samples with different CNV types (e.g. CNVs with different exons/genes/lengths) in the same sequencing run under this setting.

**DNA INPUT INFORMATION**

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

The recommended DNA input is 20-60 ng per PCR reaction for standard genomic DNA and 20-80 ng for FFPE DNA. However, if using FFPE DNA that is severely degraded, the recommended minimum input is 40 ng given the amount of material is not limited

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

**LIBRARY PREPARATION PROTOCOL**

Hands-on time: 3-5 hours
Total time: 6.5-8.5 hours

**Gene-specific PCR: Amplify Genomic DNA Targets**

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

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

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

| Reagent | Volume (uL) (without UDG) | Volume (uL) (with UDG) |
|---|---|---|
| Gene-specific PCR Master Mix | 12.5 | 12.5 |
| BR283 oligo pool | 5.0 | 5.0 |
| UDG (5 units/uL) | 0.0 | 1.0 |
| **Sub-total** | **17.5** | **18.5** |

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

2. **Transfer:** Transfer 17.5 µL (or 18.5 µL if using UDG) of PCR master mix to each sample well in a PCR plate, strip tube, or PCR tube.
3. **Dilute input DNA:** Dilute DNA in nuclease-free water to a final volume of 7.5 µL (or 6.5 µL if using UDG) of diluted DNA*. Add the diluted DNA to each sample well contain PCR master mix. Add 7.5 µL (or 6.5 µL if using UDG) of nuclease-free water to the no-template control well.

| Reagent | Volume (uL) (without UDG) | Volume (uL) (with UDG) |
|---|---|---|
| PCR Master Mix | 17.5 | 18.5 |
| Diluted DNA (or water) | 7.5 | 6.5 |
| **Sub-total** | **25.0** | **25.0** |

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

*recommended minimum input is 20 ng. However, if using FFPE DNA that is severely degraded, the recommended minimum input is 40 ng given the amount of material is not limited. Lower input amount of severely degraded FFPE may be used, but on target rate for sequencing may be low.*

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

| Temperature | Time | Number of Cycles |
|---|---|---|
| 95°C | 15 min | 1 |
| 98°C<br>58°C<br>60°C<br>64°C<br>72°C | 1 min<br>2 min<br>4 min<br>1 min<br>1 min | 5 |
| 95°C<br>66°C | 30 sec<br>3 min | 20 |
| 8°C | Hold | 1 |

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

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

**Gene-specific Primer Digestion**

Hands-on time: 10-15 minutes
Total time: 50-55 minutes

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

1. Briefly spin the reactions to remove any droplets from the side walls. Carefully remove the seal or caps.
2. **Dilute Exonuclease:**
    a. Invert the Exonuclease I to mix and spin in a microfuge to remove any droplets from the lid.
    b. For 10 samples, dilute 30 µL of the exonuclease I in 20 µL nuclease-free water to prepare 50 µL of diluted exonuclease. Add reagent overage as appropriate. Any excess dilution can be stored at -20°C overnight for a second use.

***IMPORTANT:*** *The exonuclease solution is viscous and requires careful attention when pipetting. The diluted exonuclease I is good for a second use the next day if stored overnight at -20°C. Otherwise, freshly dilute the exonuclease before adding it to the samples.*

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

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

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

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

**Gel Image After Gene-specific PCR and Primer Digestion**

The following image is an example of samples after gene-specific PCR and before primer digestion on a 2% agarose gel.

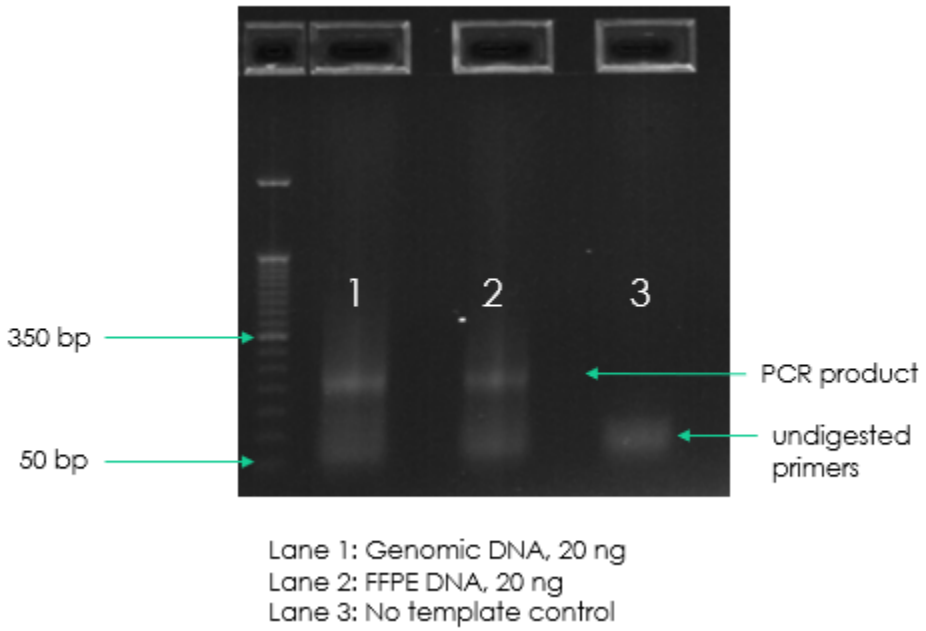


**Figure 3.** Gel analysis of GS PCR material on 2% agarose gel.

**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 nuclease-free water to each well to bring the volume to 50 µL.

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

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

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

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

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

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

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

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

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

**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 |
| **Sub-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 samples being processed with overage.

| Reagent | Volume (µL) |
|---|---|
| Indexing PCR Master Mix (2x) | 25.0 |
| Nuclease-free water | 14.0 |
| **Sub-total** | **39.0** |

3. **Add master mix to wells:** Add 39 µL prepared master mix to wells that contain indices from step 1. To prevent cross-contamination of indices, be sure to change tips between each well.

| Reagent | Volume (µL) |
|---|---|
| Pi500 and Pi700 Indices | 8.0 |
| Master Mix | 39.0 |
| **Total** | **47.0** |

4. **Add purified GS PCR product.** The following steps should be performed in a post-PCR area. ***Important***: Cover or seal the reactions before transferring from the pre-PCR area to the post-PCR area. Aliquot 3 µL of the separated

supernatant (Gene-specific PCR product) into the appropriate wells containing indices and PCR Master Mix, being sure that no beads are transferred.

| Reagent | Volume (µL) |
|---|---|
| Indices and PCR Master Mix | 47.0 |
| Gene-specific PCR product | 3.0 |
| **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<br>66°C<br>72°C | 30 sec<br>30 sec<br>60 sec | 5* |
| 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.*

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

**Purify the Libraries**

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

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

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

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

**Library Purification**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

**Final Library Image**

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

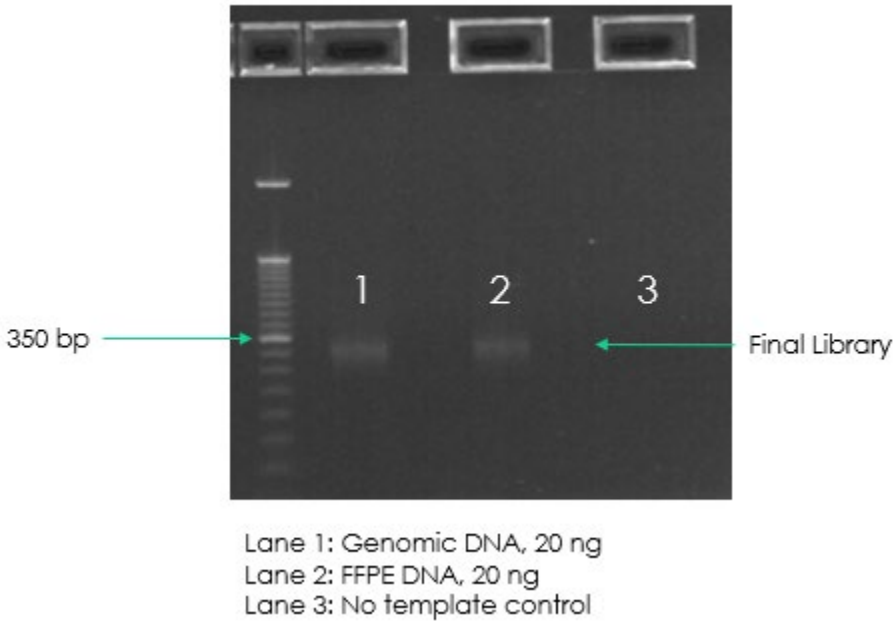


**Figure 4.** Gel analysis of final library material on a 2% agarose gel.

**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.
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

Samples can be multiplexed and sequenced on the MiSeq using the v2 or v3 chemistry, or the NextSeq using a Mid kit. The number of samples that can be loaded is dependent on the number of paired-end reads per sample and the sequencing depth. The maximum number of libraries that can be loaded is displayed in the table below (Table 1). Please choose the appropriate sequencing workflow and kit based on the number of samples to be sequenced.

| | | Maximum number of Libraries | |
|---|---|---|---|
| | | **Coverage Stats Mean: 1250** | **Coverage Stats Mean: 2500** |
| **Kit** | **Estimated PE reads** | **Min: 250** | **Min: 500** |
| MiSeq v2 Nano | 2 Million | 2 | 1 |
| MiSeq v2 Micro | 8 Million | 10 | 5 |
| MiSeq v2 | 30 Million | 38 | 19 |
| MiSeq v3 | 50 Million | 63 | 31 |
| NextSeq Mid | 260 million | 330* | 165* |

**Table 1.** Calculated estimate of the maximum number of libraries that can be loaded into each Illumina MiSeq or NextSeq kit. Calculation assumes >90% effective on-target rate after read mapping and minimum segment coverage >20% of mean coverage. (*If using Pillar Indexing primers).

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

**Sequencing on MiSeq (MiSeq v2 or v3 kit)**

For v2 or v3 chemistry (MiSeq v2 or v3 kit), dilute libraries to **5 nM.** The final concentration of the libraries for sequencing is **15.0 pM**.

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

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

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

***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 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 ONCO/Reveal™ BRCA1 & BRCA2 Somatic with CNV Panel cluster very efficiently on the MiSeq. It is recommended that the library mix be quantitated using Qubit or another library quantitation method (qPCR) to ensure the mix is at 5 nM to prevent over-clustering on the MiSeq. If the final dilution is not 5 nM (±10%), adjust the dilution in step 6 accordingly to obtain the desired concentration.*

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

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

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

5. **Denature the library mix:** Label a new 1.5 mL microtube for the denatured, 25 pM library mix.
    a. Denature the library mix by combining 5 µL of the library mix and 5 µL of the freshly prepared 0.2 N NaOH.
    b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
    c. Let the solution stand at room temperature for 5 minutes.
    d. Add 990 µL of Illumina's HT1 solution to the denatured library mix.
    e. Invert the mixture several times, spin briefly, and place on ice.
6. **Dilute to 15 pM library mix:** Label a new 1.5 mL microtube for the 15.0 pM library mix. Combine 360 µL of the 25 pM library mix (step 5) with 240 µL of Illumina's HT1 solution. Adjust the volumes as needed for libraries that are over or under 25 pM. Invert the mixture several times, spin briefly, and place on ice.
7. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 594 µL of the 15.0 pM library mix (step 6) with 6 µL of a 12.5 pM PhiX library control. Briefly vortex, spin, and place on ice.
8. **Load MiSeq cartridge:** Using a clean 1000 µL tip, puncture the foil cap above the sample loading tube on the MiSeq cartridge. Load the 600 µL library mix and PhiX mixture (step 7) into the cartridge and ensure the solution has reached the bottom of the tube by lightly tapping the tube if liquid remains on the side wall or if there is an air bubble at the bottom of the tube.
9. **Run the MiSeq:** Run the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) and two indexing reads of 8 cycles each: "MiSeq System User Guide" (part #15027617). For instructions on preparing a sample sheet for the MiSeq, see page 29.
10. Store diluted libraries and mixtures at -20°C for long-term storage.

**Sequencing on the NextSeq**

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

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

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

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

***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 4 µL of each 5 nM library). Quickly vortex the mix for 2-5 seconds and spin down.

   *It is recommended that the library mix be quantitated using Qubit or another library quantitation method (qPCR) to ensure the mix is at 5 nM to prevent over- or under-clustering on the NextSeq. If the final dilution is not 5 nM (±10%), adjust the dilution in step 6 accordingly to obtain the desired concentration.*

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

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

   Alternately, prepare a 1 N NaOH solution by combining 500 µL 10 N NaOH into 4.5 mL of nuclease-free water. Vortex the solution to mix. If 1 N NaOH

has not been prepared within the last week from a 10 N solution, prepare a new 1 N NaOH solution.

5. **Denature the library mix:** Label a new microtube for the denatured, 25 pM library mix.
    a. Denature the library mix by combining 5 µL of the library mix and 5 µL of the freshly prepared 0.2 N NaOH.
    b. Vortex the solution thoroughly for 10 seconds and centrifuge the solution in a microfuge for 1 minute.
    c. Let the solution stand at room temperature for 5 minutes.
    d. Add 5 µL of 200 mM Tris-HCl, pH 7.0.
    e. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
    f. Add 985 µL of Illumina's HT1 solution to the denatured library mix.
    g. Vortex briefly and centrifuge the solution in a microfuge for 1 minute.
6. **Dilute 25 pM library mix to 1.8 pM:** Dilute the denatured library (step 5) 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 solution. Invert to mix and spin briefly.
7. **Combine library mix and PhiX control:** Label a new 1.5 mL microtube for the mixture that will be loaded. Combine 1287 µL of the 1.8 pM library mix (step 6) with 13 µL of a 1.8 pM PhiX library control. Briefly vortex, spin, and place on ice.
8. **Load NextSeq cartridge:** Using a clean 1000 µL tip, puncture the foil cap above the sample loading well on the NextSeq cartridge. Load 1300 µL library mix and PhiX mixture (step 7) into the cartridge and ensure the solution has reached the bottom of the cartridge well.
9. **Run the NextSeq:** Run the libraries on the NextSeq per the manufacturer's instructions using a paired-end read length of 150 (2x150) and two indexing reads of 8 cycles each: "NextSeq System User Guide" (part #15046563 or 15069765). For instructions on preparing a sample sheet for the NextSeq, see page 28.
10. **Store Libraries**: Store diluted libraries and mixtures at -20°C for long-term storage.

**Preparing a Sample Sheet for Sequencing**

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

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

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

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

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

**CNV Analysis using PiVAT**

See the *PiVAT User Manual* for instructions on starting CNV analysis. For PiVAT 2020.1 version, there is no post-call CNV filters for false positives. A threshold of CNV_Call>=1.25 or CNV_Call<=0.75 is recommended to be applied by the user for Germline CNV calling. The normalized copy number can be found in 'Normalized Coverages' sheet in CNV_RESULTS.

List of user adjustable Quality Control (QC) parameters:

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.)

Please refer to 'CNV Cluster Calls' sheet in CNV_RESULTS excel file for BRCA-CNV calls and ignore the 'CNV Calls' sheet. In the table below, the column headers in the "CNV Cluster Calls' sheet is explained.

**"CNV Cluster Calls" Sheet Column Explanation:**

| | |
|---|---|
| **Sample_ID** | Unique Sample ID for each sample |
| **CNV_Cluster** | A numerical ID number for the called cluster of amplicons. |
| **CNV_Call** | Average copy number ratio with respect to normal copy number (2) of the called cluster of amplicons. |
| **ZScore** | Calculated Z-score of the cluster CNV call. |
| **Gene_ID** | Simplified gene names for the amplicons in the called cluster. |
| **Cov_Norm_Mean** | Mean of the normalized copy number ratios for the amplicons in the called cluster. Same as 'CNV Call' column. |
| **Cov_Norm_Std** | Standard deviation of the normalized copy number ratios for the amplicons in the called cluster. |
| **Cov_Norm_List** | List of the normalized copy number ratios for the amplicons in the called cluster. |
| **Amp_Ct** | Count of amplicons in the called cluster. |
| **Target_Name** | List of the amplicon names in the called cluster. |

**TROUBLESHOOTING**

| Issue | Potential Cause | Solution |
|---|---|---|
| Low yield of gene-specific product | DNA quantity or quality | The recommended input for the assay is 20 ng of genomic DNA or 40 ng for FFPE DNA. Higher quantities may be necessary for low- or poor-quality samples. |
| | Improper cycling | Check that the cycling protocol performed is the appropriate protocol for gene-specific amplification. |
| Low indexing efficiency | Improper AMPure purification | Incomplete AMPure purification or loss of gene-specific product will affect the indexing PCR reaction. The purified product can be checked on an agarose gel to ensure the gene-specific product was not lost or that clean-up was sufficient to remove excess primers. |
| | | The AMPure bead ratio and ethanol concentration affect the PCR cleanup. Ensure the correct AMPure concentration was used for cleanup and fresh, 70% ethanol is used for the wash. |
| | | Leftover ethanol from the wash steps can hinder the PCR reaction. Remove as much of the ethanol during the final wash step with a pipette and dry the beads to ensure the residual ethanol has evaporated. |
| | Partial primer digestion | Poor digestion of the gene-specific primers can hinder the indexing efficiency of the indexing PCR reaction. Check the primer digestion using an agarose gel. |
| | Incomplete deactivation of exonuclease | The inactivation of the nuclease and AMPure purification is necessary before performing indexing PCR. |

| | | |
|---|---|---|
| | | Leftover active exonuclease can digest the indexing PCR primers, reducing the yield of the indexing PCR reaction. |
| Low library yield | DNA quantity or quality | The recommended input for the assay is 20 ng of genomic DNA or 40 ng for FFPE DNA. Higher quantities may be necessary for low- or poor-quality samples.<br><br>Run the product from the gene-specific PCR on agarose gel to check the yield.<br><br>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.<br><br>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 15 pM (MiSeq), or 1.8 pM (NextSeq) | Check the 5 nM library mix using Qubit or RT-PCR. Dilute the denatured library mix as needed to adjust for the difference in concentration. |
| | Improper library quantitation | Improper library quantitation may result in artificially high or low yields, which affects downstream normalization. |

| | | |
|---|---|---|
| | | Re-quantitate the final libraries and/or the normalized libraries to check for the expected values. |
| | Improper AMPure purification | Changing the ratio of AMPure beads affects the purification of the products. Notably, the presence of primer dimers can cause an underestimation of total quantity, causing over-clustering.<br><br>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.<br><br>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.<br><br>Work spaces and equipment for pre-PCR and post-PCR should be separated to prevent amplicon contamination.<br><br>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. |