oncoReveal™ Core LBx

USER MANUAL

UM-0075 version 1.0
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Revision History

Version 1.0: Initial Release

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1. Product Introduction

The **oncoReveal™ Core LBx Panel** is a robust NGS assay that interrogates 446 targets across 104 genes of interest from multiple solid tumor cancer types. The panel can detect four types of variants from cell-free DNA (cfDNA) from plasma: single nucleotide variants (SNVs), small insertion/deletion (indel) variants, copy number amplification (CNA), and microsatellite instability (MSI). For more information on the panel specifications, please see the oncoReveal™ Core LBx product sheet, MK-0044.

The panel is designed to differentiate between variant signals that originate from tumor cells and healthy normal cells. This requires the use of normal cfDNA negative control samples. The detection of low-frequency variants and CNAs is achieved by modeling and accounting for the noise and amplification profile of the negative controls. The choice of negative control samples and how those control samples are run can impact the results. For more information, please refer to Section 8: *Negative Control Requirements and Variant Calling with PiVAT*.

2. Product Description

The oncoReveal™ Core LBx Panel utilizes our SLIMamp® (stem-loop inhibition mediated amplification) technology with a single-tube multiplex PCR reaction. A pair of DNA oligos designed for each region of interest, or hotspot, is used in the first round of gene-specific PCR (GS-PCR), and the products are subsequently purified via size selection. The oncoReveal™ Core LBx Panel contains sufficient reagents to prepare 24 libraries. The panel requires a unique dual barcode indexing kit (available separately; see section 4.2) and the resulting libraries are designed for sequencing on the Illumina platform using a paired-end read length of at least 2x121. Please note that as the average panel amplicon size is 80 bp; sequencing greater than 121 bp may lead to lower read quality scores without affecting downstream data analysis by PiVAT.

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.

To identify SNV/indel variants down to 0.1% frequency with maximum sensitivity and specificity, we recommend starting with cell-free DNA (cfDNA) input greater or equal to 20 ng. Input DNA below 10 ng will amplify but with decreased sensitivity of detection.

Before using the kit, it is essential to review sections 7 and 8 that describe the loading schemes and the selection of negative control samples, respectively. Note that SNV/Indel and CNA controls can be used as in-lot controls for subsequent runs within the same kit lots.

3. Guidelines

3.1 cfDNA Sample Preparation and QC Guidelines

- **Blood collection:** This panel was developed using Streck Cell-Free DNA BCT® tubes *(Cat. No. 230469, 230470, 230471)*. Plasma should be isolated according to the manufacturer's guidelines. Plasma can be isolated up to 7 days after blood draw when the tubes are stored at 18° to 25°C. The Streck Cell-Free DNA BCT tube has a capacity of 10 mL. Isolation of plasma from a full blood draw will typically produce between 4 and 5 mL; however, yields may vary due to differences between individual donors. Performance characteristics of other collection tube types, such as K_2EDTA, have not been evaluated with this assay.

- **cfDNA extraction:** The oncoReveal™ Core LBx panel was developed and extensively tested using cfDNA extracted with the Qiagen QIAamp Circulating Nucleic Acid Kit *(Cat. No. 55114)*. Other extraction methods may be used; however their performance has not been assessed and results may vary.

- **Input volume:** The QIAamp Circulating Nucleic Acid Kit is compatible with sample volumes from 1 to 5 mL. To increase cfDNA yield, extraction from either 4 or 5 mL of plasma is recommended.

- **Elution volume:** The elution volume of the QIAamp Circulating Nucleic Acid Kit is 20 to 150 µL. An elution volume of 30 µL is recommended to increase cfDNA concentration.

- **Cross-contamination:** The oncoReveal™ Core LBx Panel is extremely sensitive, allowing for the detection of variants down to 0.1% allele frequencies. As such, it is crucial that care must be taken during all steps of sample preparation to avoid inaccuracies in downstream results arising from pre-analytical errors. Consult section 3.2 for more details.

- **Quantification:** Quantification using the Invitrogen Qubit dsDNA High Sensitivity Assay kit *(Thermo Fisher Scientific, Cat. No. Q32851, Q32854)* is recommended for cfDNA extracted using the QIAamp Circulating Nucleic Acid Kit. Qubit dsDNA High Sensitivity Assay kit is highly selective for double-stranded DNA. Due to the presence of carrier RNA in the eluate, quantification with spectrophotometry or with fluorometric methods that utilize single-stranded binding dyes may cause overestimation of cfDNA quantity.

- **Genomic DNA (gDNA) contamination:** gDNA contamination is caused by the lysis of white blood cells in either the blood or plasma sample. The presence of gDNA contamination in cfDNA extracts may cause underestimation of variants or false negative results, especially for variants present at low allele frequencies. The manufacturer's guidelines for sample collection and processing should be followed carefully to minimize gDNA contamination. Additionally, several QC methods are recommended to check for gDNA contamination:

  - *Gel Electrophoresis*: Typical cfDNA samples will contain fragments between 50 and 700 bp in size, with one major band occurring around 150 bp and a second major band around 300 bp. Observation of bands or smearing above 700 bp indicates the presence of gDNA contamination.

  - *TapeStation*: Sample QC with Agilent Cell-free DNA ScreenTape *(Cat. No. 5067-5630)* provides a measurement of %cfDNA. Lower %cfDNA values indicate the presence of contaminating gDNA.

  - *qPCR*: A qPCR method utilizing amplicons of varying fragment sizes may be used for both cfDNA quantification and assessment of gDNA contamination. This method is particularly useful if alternative

cfDNA extraction kits are used.

- To identify SNV/indel variants down to 0.1% frequency with maximum sensitivity and specificity, we recommend starting with cell-free DNA (cfDNA) input greater or equal to 20 ng with a target of least 16.7 M clusters. Input DNA below 10 ng will amplify but with decreased sensitivity of detection.

3.2   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 with 70% alcohol or freshly-made 10% hypochlorite solution. Gloves should be changed after handling a contaminated item.

- **Aliquot reagents:** Frozen reagents should be aliquoted 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, the 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

oncoReveal™ Core LBx Panel Part No.: HLA-HS-1004-24

Reagent	Use	Area Use	Storage
PCR HiFi 4x Master Mix	Gene-Specific PCR and Indexing PCR	Pre-PCR	-25° to -15°C
oncoReveal™ Core LBx Oligo Pool	Gene-Specific PCR	Pre-PCR	-25° to -15°C
GC Rescue G	Gene-Specific PCR	Pre-PCR	-25° to -15°C
Magnesium chloride	Gene-Specific PCR	Pre-PCR	-25° to -15°C

4.2 Liquid Biopsy (LBx) Unique Dual Indexing kits

Reagent	Part Number	Use	Area Use	Storage
Pillar Biosciences LBx Indexing Kit A	IDX-PI-1013-96	Indexing PCR	Pre-PCR	-25° to -15°C
Pillar Biosciences LBx Indexing Kit B	IDX-PI-1014-96	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
Negative control cfDNA	Pre-PCR	Refer to Section 8 of this manual
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
Agarose gel, 2% (optional) [1]	Post-PCR	General lab supplier
DNA molecular weight markers (optional) [1]	Post-PCR	General lab supplier
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
200 mM Tris-HCl, pH 7.0	Post-PCR	General lab supplier

[1] *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 an agarose gel or using the TapeStation DNA Analysis kit.*

Compatible Sequencing Reagents

Sequencing Reagent Kit	Supplier	Catalog No.
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, NextSeq 2000, NovaSeq 6000	Illumina	Refer to Illumina's website

General Laboratory Consumables

In addition to the consumables listed below other general laboratory supplies needed to carry out the protocol include gloves, ice buckets, 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

4.4 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 an agarose gel.

5. Workflow

The following chart demonstrates the workflow for performing the oncoReveal™ Core LBx Panel library preparation.

Gene-Specific PCR

Hands-on time: 30 - 45 min.
Total time: 3.25 – 3.75 hours

Purify PCR Product

Hands-on time: 60 - 90 min.
Total time: 60 - 90 min.

Indexing PCR

Hands-on time: 20 - 40 min.
Total time: 50 - 70 min.

Purify Libraries

Hands-on time: 30 - 45 min.
Total time: 30 - 45 min.

Quantify and Load Libraries

Hands-on time: 1 - 1.5 hours
Total time: 1.5 - 2 hours

Pre- PCR

Post-PCR

Stopping Point (Optional)

Hands-on time:
3 – 5 hours
Total time:
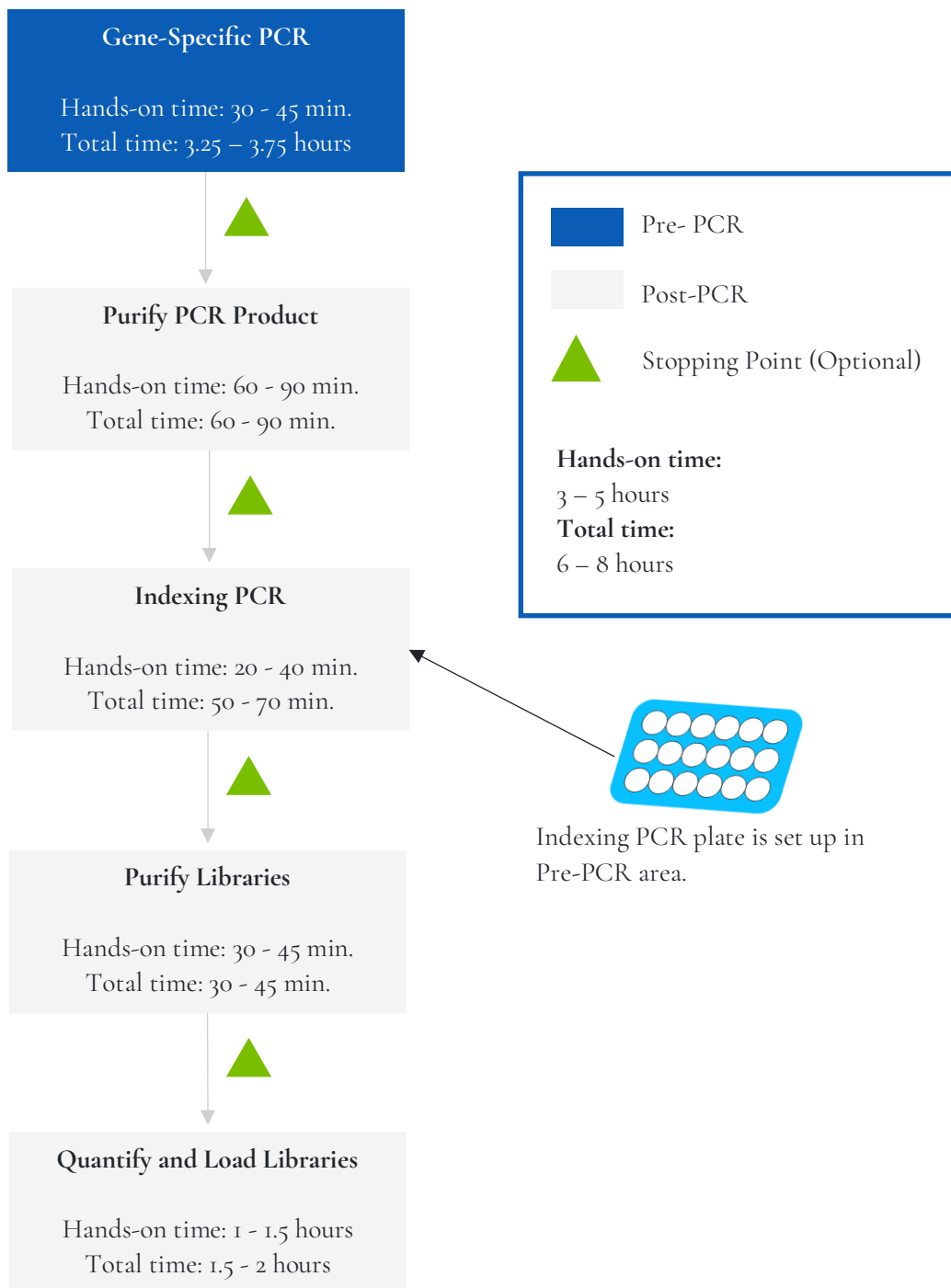6 – 8 hours

Indexing PCR plate is set up in Pre-PCR area.

Figure 1. Library preparation workflow for oncoReveal™ Core LBx Panel. The workflow can be completed within a day but contains multiple optional stopping points for users with time constraints.

Symbol	Description
![info]	**Information:** Information that follows this symbol is important and may require action.
![stop]	**Optional Stopping Point:** A point in the workflow at which work may be safely paused, and samples can be stored appropriately.
![caution]	**Caution:** 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.

6. Library Preparation Protocol

Hands-on Time	3 – 5 hours
Total Time	6.5 – 8 hours

6.1 Gene-Specific Target Amplification (GS-PCR) & Target Amplicon Purification

Hands-on Time	30 – 40 min.
Total Time	2.75 – 3 hours

Amplify cfDNA Targets

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

For this portion of the protocol prepare a prechilled cooler and keep the 4x HiFi Master Mix, the custom oligo pool, and GC Rescue G on ice.

1. **Prepare Gene-Specific PCR Mix 1 (GS-PCR Mix 1):** Vortex and centrifuge the 4x HiFi Master Mix and the oligo pool before use. For each PCR reaction, the volume of each component is listed below.

![info] **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 HiFi 4x Master Mix	12.5
Core LBx Oligo Pool	2.2
GC Rescue G	2.5
Magnesium Chloride	1.0
Subtotal	**18.2**

2. **Transfer:** Transfer **18.2 µL** of GS-PCR Mix 1 to each sample well in a PCR plate, strip tube, or PCR tube.

3. **Dilute input DNA**: Add **31.8 µL** of DNA (diluted if necessary) to each sample well containing GS-PCR Mix. Add **31.8 µL** of nuclease-free water to the no-template control well.

![info]

When performing an in-batch normal run, cfDNA (≥20 ng) from a healthy individual is recommended as input, for a target of at least **66M single-end (33M paired-end) reads**. Note that this will reduce the test sample capacity in the flowcell by 2. Please see Sections 7 and 8 for further information.

The recommended input range is 10 – 30 ng of cfDNA.

Final GS-PCR Mix	
Reagent	**Volume (µL)**
GS-PCR Mix 1	18.2
Diluted DNA (or water)	31.8
Total	**50.0**

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

4. **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 sec	1
95°C	1 min	
58°C	2 min	
60°C	4 min	10
64°C	1 min	
72°C	1 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.

Purify the Gene-Specific PCR Product

Hands-on Time	60 – 90 min.
Total Time	60 – 90 min.

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

Before beginning the purification:

- Remove the 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

1. If condensation has formed or if reactions were stored at -20°C briefly centrifuge the reactions upon thawing. Carefully remove the seal.

2. **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.

3. **Add beads:** Add **75 µL** of AMPure beads (equivalent to a 1.5x 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.

4. **Bind GS-PCR product to beads:** Incubate for 5 minutes at room temperature.

5. **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.

6. **Remove supernatant:** Carefully remove the supernatant from each well without disturbing the beads.

7. **Resuspend beads:** Remove the PCR plate from the magnetic rack and resuspend the dried beads in each well using **53 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.

8. **Incubate:** Incubate for 5 minutes at room temperature.

9. **Separate GS-PCR product from beads:** Place the PCR plate on a magnetic rack at room temperature until the solution appears clear. Transfer **50 μL** of clear supernatant from each well of the PCR plate to a new PCR plate.

10. **Second Cleanup:** With the eluted product from step 9, repeat steps 3-6 for a second round of purification.

11. **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.

12. **Second wash:** Repeat step 11 for a second 70% ethanol wash. Remove the supernatant from each well.

13. **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 the remaining ethanol solution at the bottom of the wells.

14. **Dry beads:** Keep the PCR plate 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.

15. **Resuspend Beads:** Remove the PCR plate 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.

16. **Incubate and Elute:** Incubate for 5 minutes at room temperature.

17. **Separate GS-PCR product from beads:** Place the PCR plate on a magnetic rack at room temperature until the solution appears clear. Transfer **30 μL** of clear supernatant from each well of the PCR plate to a new PCR plate.

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

6.2   Index PCR & Purification

Indexing PCR

Hands-on Time	20 – 40 min.
Total Time	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.

1.   **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 HiFi Master Mix and water with sufficient overage.

Indexing PCR Mix 1 (per reaction)	
Reagent	**Volume (μL)**
4x HiFi Master Mix	12.5
Nuclease-free water	23.5
Subtotal	**36.0**

2.   Transfer **36 μL** of the Indexing PCR Mix 1 to each sample well of a PCR plate.

3.   Transfer **8uL** of unique, pre-mixed Index Mix P5xx & P7xx to each sample well of the PCR plate. Ensure no well-to-well cross-contamination during indexing primer transfer.

Indexing PCR Mix 2 (per reaction)	
Reagent	**Volume (μL)**
Indexing PCR Mix 1	36.0
Index Mix P5xx & P7xx	8.0
Subtotal	**44.0**

> **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 the samples have been thawed at room temperature thoroughly before proceeding.

1.   **Add purified GS-PCR product:** Add **6 μL** of purified GS-PCR product into the appropriate wells containing Indexing PCR Mix 2.

Indexing PCR Final Mix (per reaction)	
Reagent	**Volume (µL)**
Indexing PCR Mix 2	44.0
Purified Gene-Specific PCR product	6.0
Total	**50.0**

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

3. **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.

4. **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	
66°C	30 sec	18*
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.

Indexing PCR: Purify the Libraries

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

Before beginning the purification:

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

- 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. 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.

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 **60 µL** 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 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 µL 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 µL** 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 µL** of clear supernatant from each well of the PCR plate 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 4ºC for up to 3 days, or at -20ºC for longer-term storage.

6.3 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

Hands-on Time	30 – 45 min.
Total Time	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 µL is sufficient buffer for 10 readings (8 samples + 2 standards). Combine 1990 µL of Qubit dsDNA HS buffer and 10 µL HS reagent. Add reagent overage appropriately.

   Important: Fluorescent dyes are sensitive to light. Protect the Qubit working solution from light.

2. **Label tubes:** Set up 0.5 mL Qubit tubes for standards and samples. Label the tube lids.

3. **Prepare standards**: Transfer **190 µL** of Qubit working solution into two tubes for standard 1 and standard 2, and then add **10 µL** of each standard to the corresponding tube.

   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 µL** of Qubit working solution to each 0.5 mL tube, and then add **2 µL** 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 µL Qubit working solution and 1 µL library (1:200 dilution). Repeat steps 5–7.

8. **Calculate concentration**: 1 ng/µL of library is equal to **6 nM**. Example calculation is below. Adjust dilution factor accordingly.

   2 µL of library + 198 µL Qubit working solution:

   $$\frac{Qubit\ reading\ \left(\frac{ng}{mL}\right)}{1,000} \times dilution\ factor\ (100) \times conversion\ factor\ (6) = nM$$

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

Hands-on Time	30 – 45 min.
Total Time	30 – 45 min.

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

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. For NTCs, use the same dilution scheme as the library with the lowest measured concentration. An example calculation is as follows:

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

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

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.

Important: If the in-batch normal sample library was included, mix **2x** volume of the in-batch normal library with the other libraries (e.g., 10 µL of normal library and 5 µL of each 5 nM library)

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 (± 10%) to prevent poor sequencing performance. If the final dilution is not 5 nM (±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.

7. Sequencing

Prepare Diluted Libraries for Sequencing

Hands-on Time	30 – 40 min.
Total Time	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 which 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. An example of the loading scheme is shown in Table 3.

Sequencing Configuration	Index	Recommended Coverage	Recommended Reads Per Sample
2 x 121	Unique dual indices (8 bp each)	Mean: 33,000X	33 million PE reads

Table 1. Sequencing recommendations.

Sequencing Flowcell	Estimated Instrument Output Paired-End Reads	Estimated Maximum Sequencing Batch Size
NextSeq 500/550 Mid Output	260	7
NextSeq 500/550 High Output	800	21

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.

Within-batch control run		Library pooling volume	Out-of-batch control run	
NextSeq Mid	**NextSeq High**	**Library pooling volume**	**NextSeq Mid**	**NextSeq High**
Negative control	Negative control	8 μL	N/A	N/A
NTC (optional)	NTC (optional)	4 μL	NTC (optional)	NTC (optional)
Sample1	Sample1	4 μL	Sample1	Sample1
Sample2	Sample2	4 μL	Sample2	Sample2
Sample3	Sample3	4 μL	Sample3	Sample3
Sample4	Sample4	4 μL	Sample4	Sample4
Sample5	Sample5	4 μL	Sample5	Sample5
	Sample6	4 μL	Sample6	Sample6
	Sample7	4 μL	Sample7	Sample7

Within-batch control run		Library pooling volume	Out-of-batch control run	
NextSeq Mid	NextSeq High		NextSeq Mid	NextSeq High
	Sample8	4 µL		Sample8
	Sample9	4 µL		Sample9
	Sample10	4 µL		Sample10
	Sample11	4 µL		Sample11
	Sample12	4 µL		Sample12
	Sample13	4 µL		Sample13
	Sample14	4 µL		Sample14
	Sample15	4 µL		Sample15
	Sample16	4 µL		Sample16
	Sample17	4 µL		Sample17
	Sample18	4 µL		Sample18
	Sample19	4 µL		Sample19
	Sample20	4 µL		Sample20
	Sample21	4 µL		Sample21
	Sample22	4 µL		Sample22

Table 3. Loading scheme for including an in-lot control, versus out-of-batch control example.

<div style="border:1px solid #000;padding:8px;background:#e0e0e0;text-align:center;">**The following steps should be performed in a post-PCR area.**</div>

For this portion of the protocol have a prechilled benchtop cooler prepared.

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.

   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 **5 μL** of 200 mM Tris-HCl, pH 7.0.
   e. Vortex briefly and centrifuge for 1 minute.
   f. Add **985 μL** of Illumina's HT1 hybridization buffer to the denatured library mix.
   g. 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 121 (2x121) 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 sequencing reagent 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. The Pillar sample sheet generator will automatically populate the correct barcode sequence when the indexing primer is selected.

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 sequencing instrument used.

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".

8. Negative Control Requirements and Variant Calling with PiVAT®

The oncoReveal™ Core LBx Panel identifies four types of variants: single nucleotide variants (SNVs), small insertion/deletion (indels), copy number amplification (CNA), and microsatellite instability (MSI). The panel is designed to facilitate downstream analysis in PiVAT, differentiating between variant signals originating from tumor cells and healthy normal cells. PiVAT does so by utilizing a negative control sample and control regions in the panel. PiVAT uses sequencing data from the negative control to model and remove noise. The choice of negative control, and how those control samples are loaded, impact your results.

8.1 Choosing a Negative Control Sample

A negative control sample is any healthy individual cfDNA or contrived cfDNA sample that is free of somatic variants, including CNA. Note that FFPE tissue samples are not suitable to serve as a negative control, as the process of formalin fixation introduces DNA damage.

Young healthy individual cfDNA samples are the best choice for the negative control as they are the closest match possible to a subject's cfDNA. While we do recommend healthy individual cfDNA for the negative control, contrived normal cfDNA samples may also be used. The performance difference compared to healthy individual cfDNA is negligible for SNV/Indel calling, but significant for CNA calling. If a negative control is not provided, SNV and indel variant calls will still be generated, but with lower specificity, and CNA calls will not be generated. For improved CNA calling, 3 healthy individual cfDNA negative control samples are recommended. CNA calling improves slightly with additional negative controls, up to 5 as capacity will allow.

Recommendation: Healthy individual cfDNA sample with ≥20 ng, loaded with a target of at least 33 million clusters. If no healthy individual samples are available, contrived samples may be used as a replacement. Internal testing has shown that Anchor Molecular Normal cfDNA *(Part No. MS-0293)* allows for good SNV and indel calling, but relatively poor CNA calling (see table of recommendations below).

Including negative control samples in your run

Negative controls can be generated and sequenced with each run (in-batch control) or generated once per reagent lot and used repeatedly for analysis (out-of-batch control). If both options are viable, in-batch controls are recommended.

Recommendation: Healthy individual cfDNA run in a single batch and used as an out-of-batch negative control for future runs within the same reagent lot.

Can MSI matched normal samples be used as negative controls?

Matched normal samples provide the highest sensitivity results for MSI calling. However, these matched samples cannot not be used as negative controls for SNV/indel or CNA calling as they fail to capture the profile of cfDNA; only healthy or contrived cfDNA samples are suitable as a negative control.

Recommended negative controls

	Recommended	Acceptable	Poor
SNV/Indel only	Healthy cfDNA (1)	Contrived cfDNA (1)	
CNA only	Healthy cfDNA (3)	Healthy cfDNA (1)	Contrived cfDNA (1)
SNV/Indel + CNA	Healthy cfDNA (3)	Healthy cfDNA (1)	Contrived cfDNA (1)

Note that PiVAT does not perform matched normal MSI calling and SNV/indel or CNA calling in the same run. If profiling of both types of variants is desired, the same run must be queued twice: once with matched MSI calling only and once with SNV/indel and CNA calling.

8.2 Starting Your PiVAT Run

You can specify your negative control samples in PiVAT using the following steps:

1. Set up your analysis run by selecting the correct panel type and FASTQ input. The negative control FASTQ files should be selected as well.
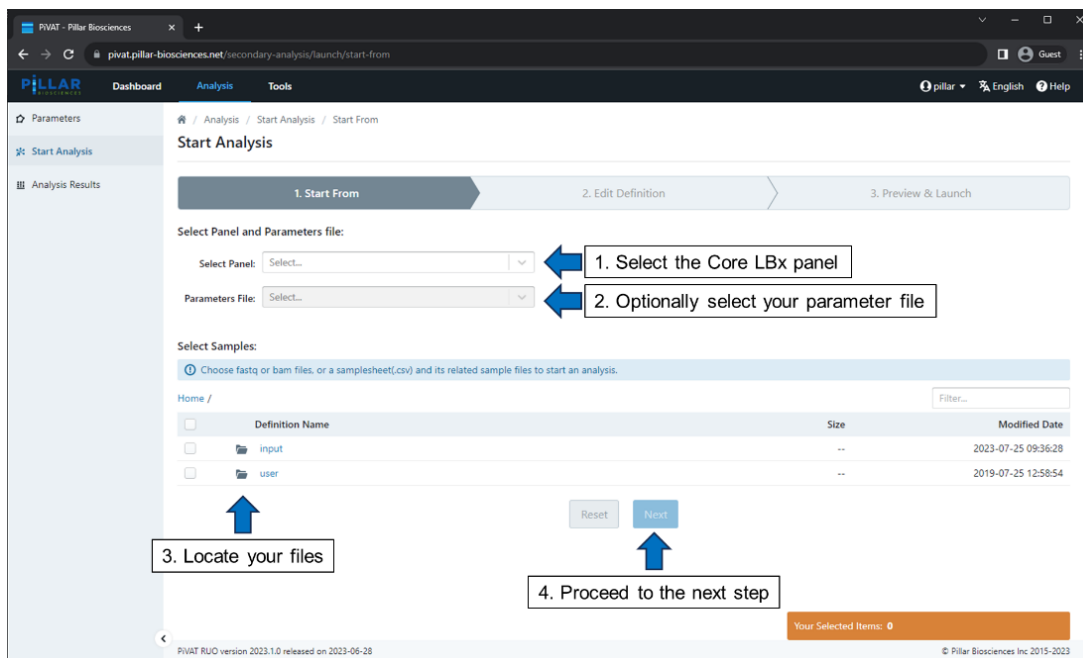

2. For SNV/indel, specify your negative control sample by selecting "NegCtrl" from the drop down under the "QC Type" column.
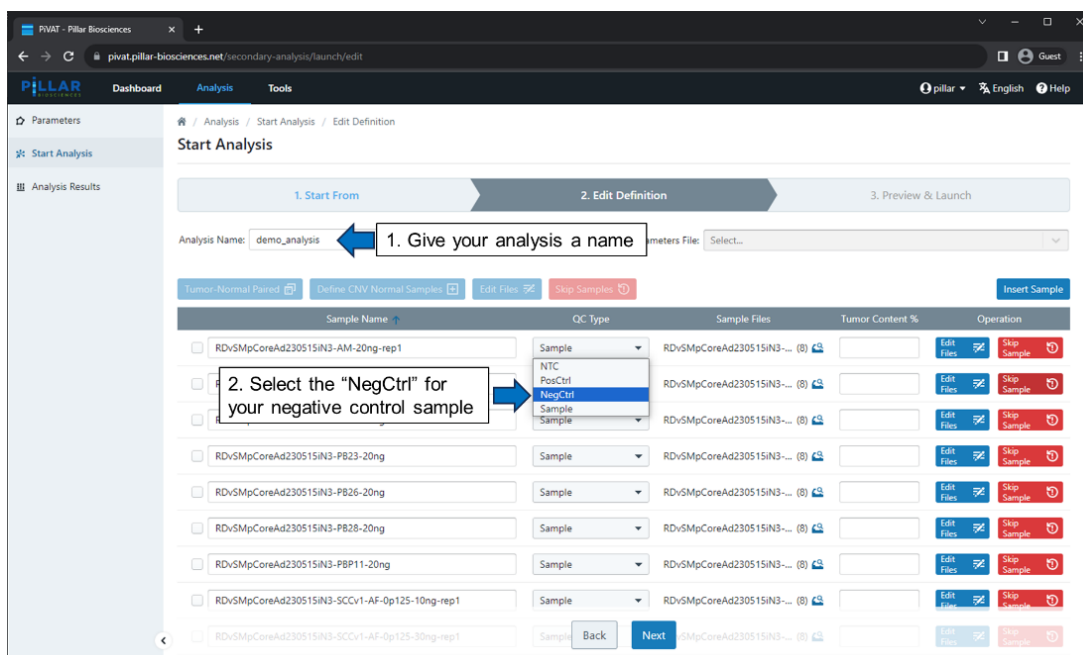
3. For CNA calling, select your sample first, then click on the "Define CNV Normal Samples" button above the sample table.
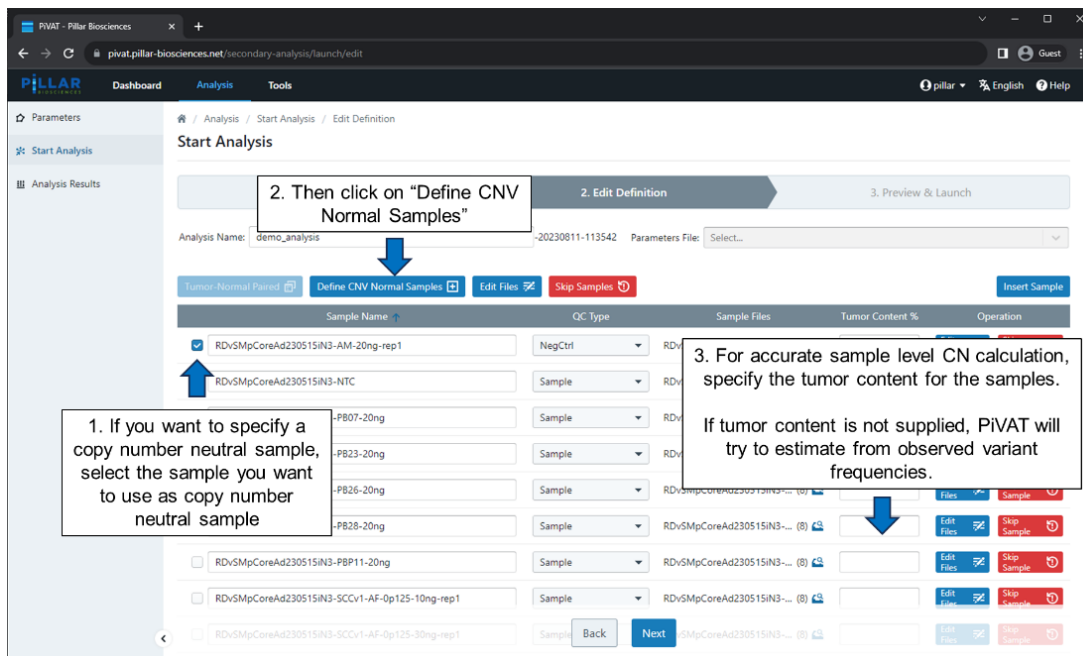

Please note that the term "Normal Sample" is identical to "Negative Control" sample. For performing both SNV/indel and CNA calling, the same sample can be used as negative control for both. This will move your sample to the bottom of the table under "Normal Samples":
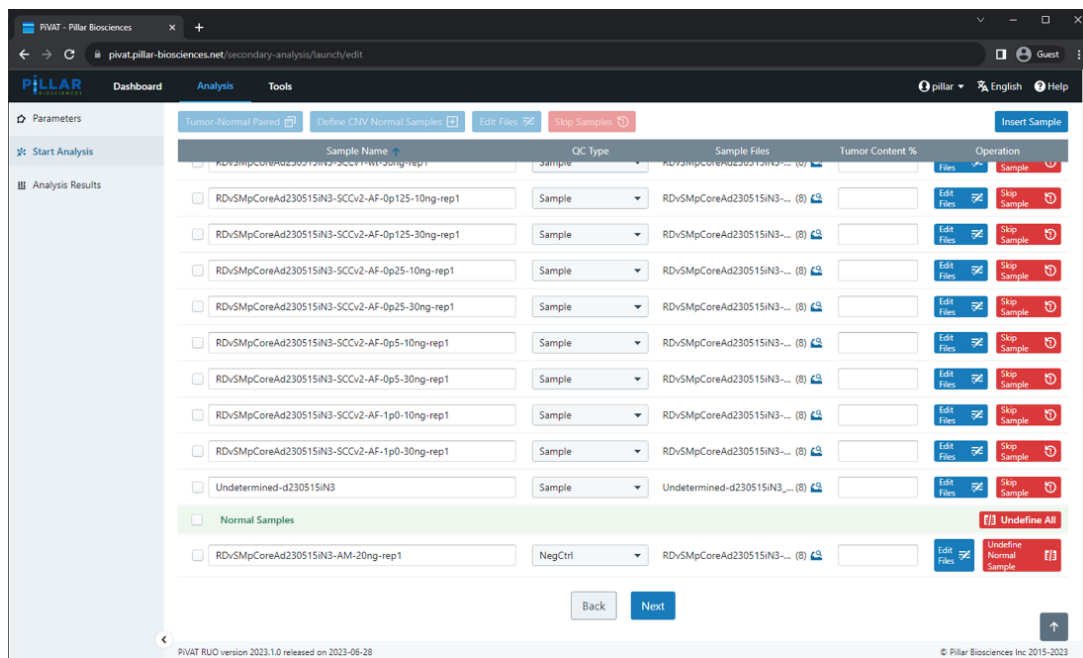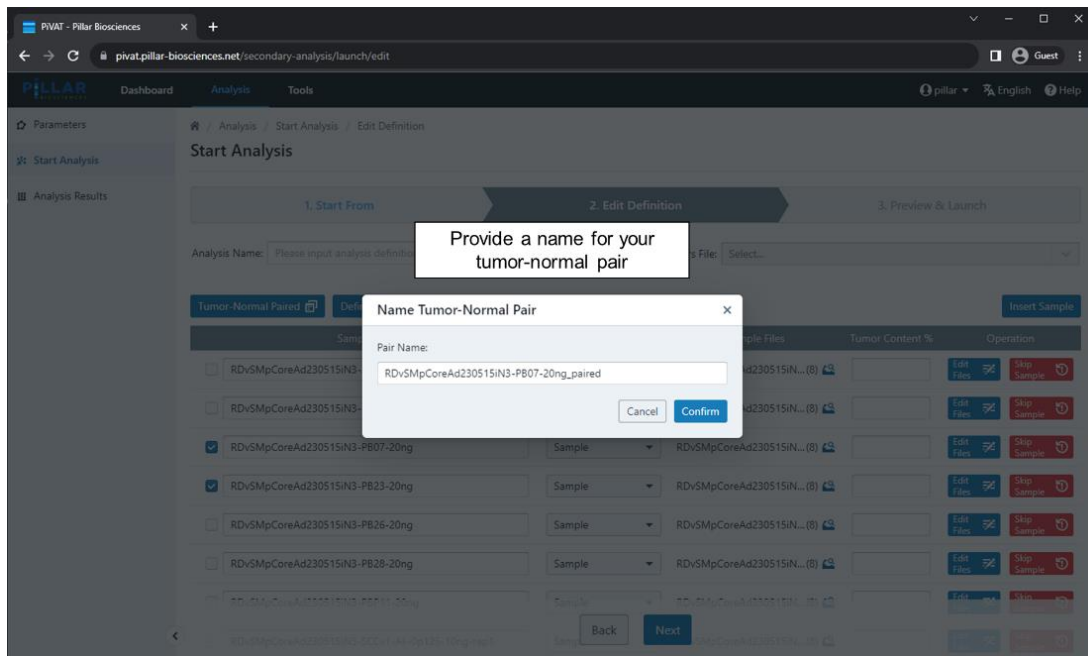
4. If matched normal MSI calling run is needed, please set up in the following manner.
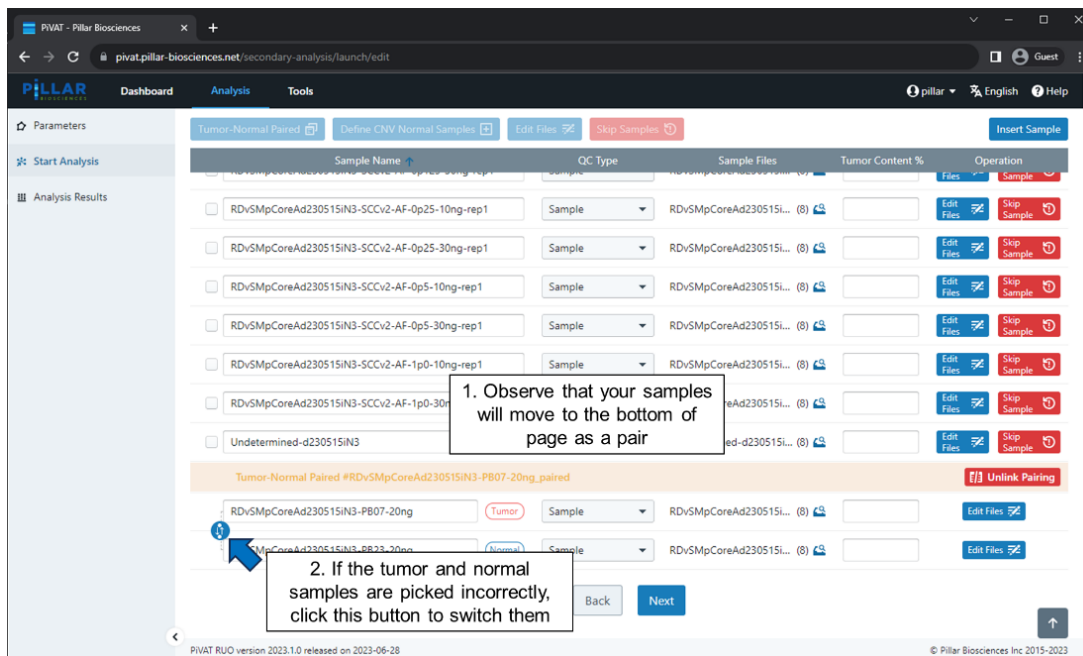
    Please note that matched normal MSI calling and CNA calling are mutually exclusive calling modes. PiVAT will only run in one of the two calling modes in a single run.
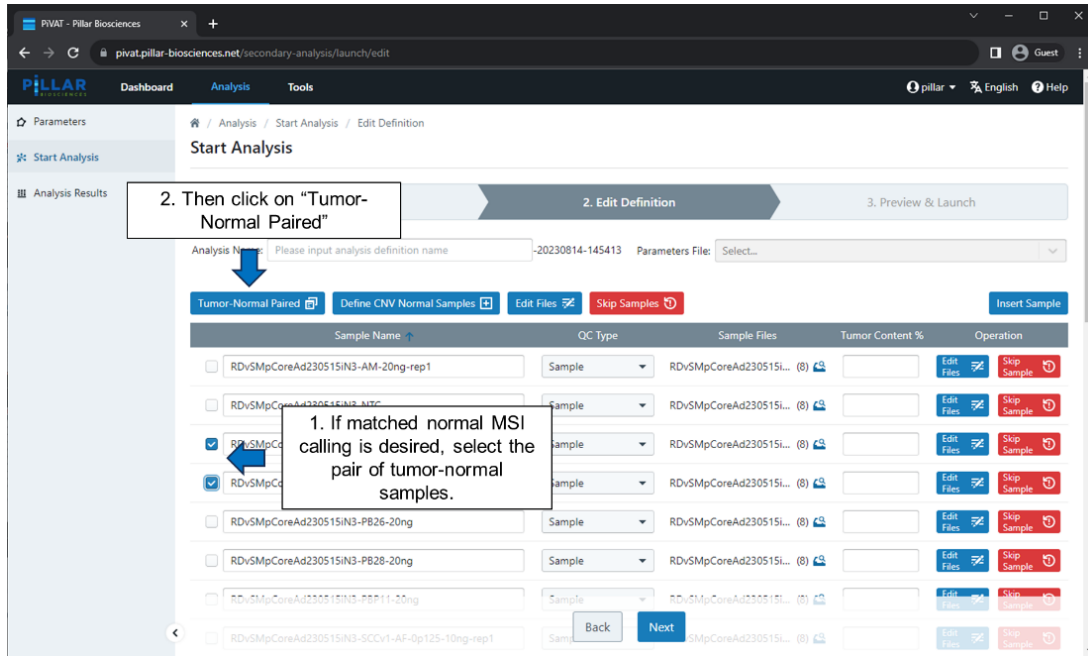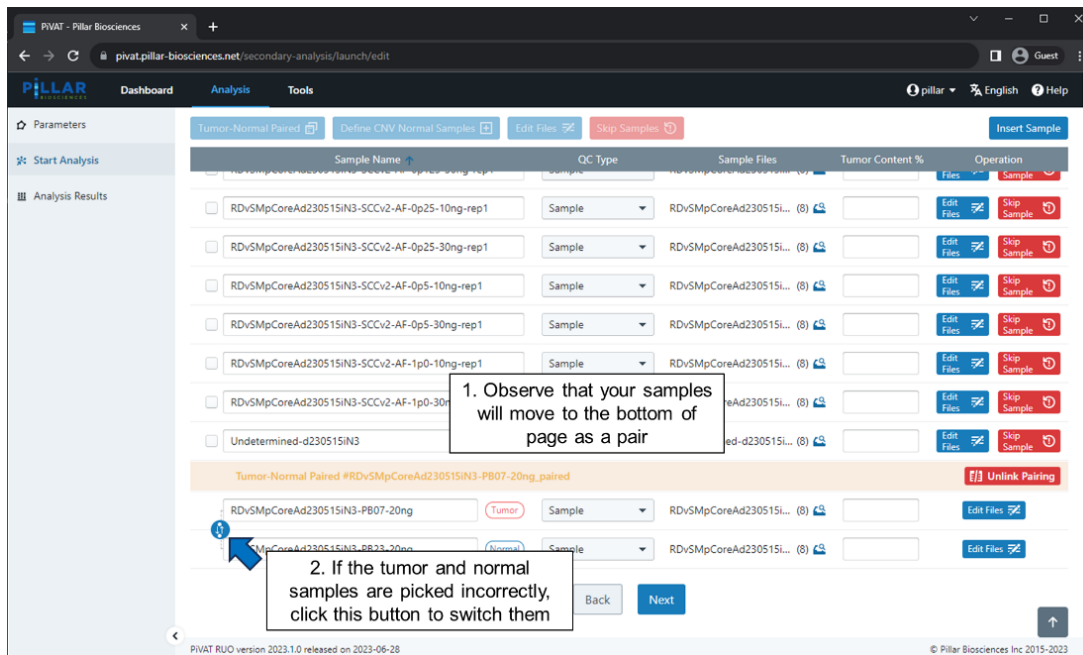

5. Enter a name for the matched pair.

Once the name has been selected, it will move your sample to the bottom of the table under "Tumor-Normal Paired SAMPLE_NAME":

6. Verify if the tumor and normal sample selection is accurate:

7.   Verify the run set up in the last window. Remember to scroll all the way down and confirm that your negative control samples are reflected appropriately.
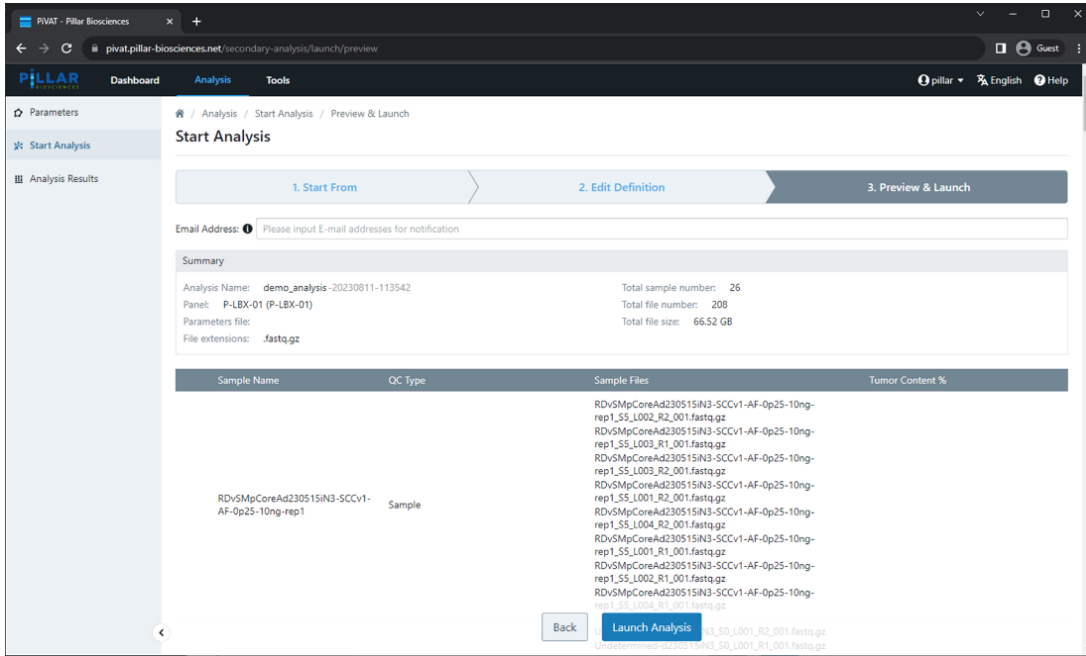
9. Troubleshooting Library Preparation

Low Yield of Gene-Specific Product

Potential Cause	Solution
DNA quantity or quality	The recommended input for the assay is 10–30 ng of cell free 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

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 10–30 ng of cell free DNA. 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.
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 that the product was not lost during PCR clean-up.

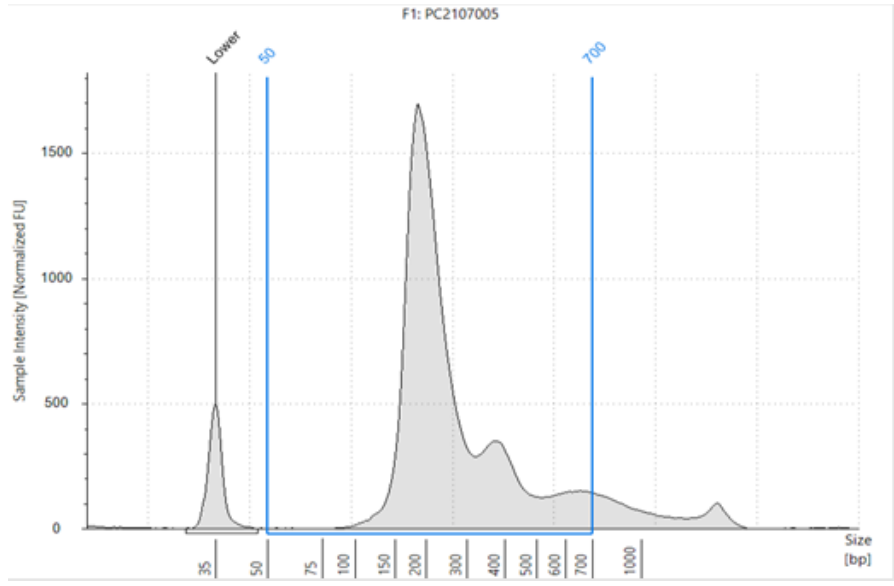|	The AMPure bead ratio and ethanol concentration affect the PCR clean-up. Ensure that the correct AMPure bead concentration was used for clean-up, and fresh 70% ethanol was used for the wash.

Amplicon Contamination in No-Template Control

| Potential Cause	Solution
Cross-contamination	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
Improper normalization and pooling of libraries	Confirm that the appropriate loading concentration was used for the applicable sequencing instrument.
	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.

	Changing the ratio of AMPure beads affects the purification of the products. Notably, the presence of primer dimers can cause an underestimation of total quantity, causing over-clustering. The final libraries can be checked on an agarose gel for the proper product size and presence of primer dimers.
Improper AMPure purification	
	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.

Few or No Somatic Variant Calls

Potential Cause	Solution
Low input sample	The oncoReveal™ Core LBx Panel is designed for input of at least 10 ng cfDNA. Samples below the recommended input range (10-30 ng) will have significantly lower sensitivity.
Low coverage or low input negative control	Check that the negative control was sequenced to at least 66 million single-end (33 million paired-end) reads and was at least 20 ng healthy individual cfDNA.
Genomic DNA (gDNA) Contamination	gDNA contamination may cause underestimation of variants or false negative results, especially for variants present at low allele frequencies. Verify that the sample does not have high levels of gDNA contamination. Example of cell-free DNA TapeStation analysis demonstrating low gDNA contamination.

Example of cell-free DNA TapeStation analysis demonstrating high gDNA contamination.
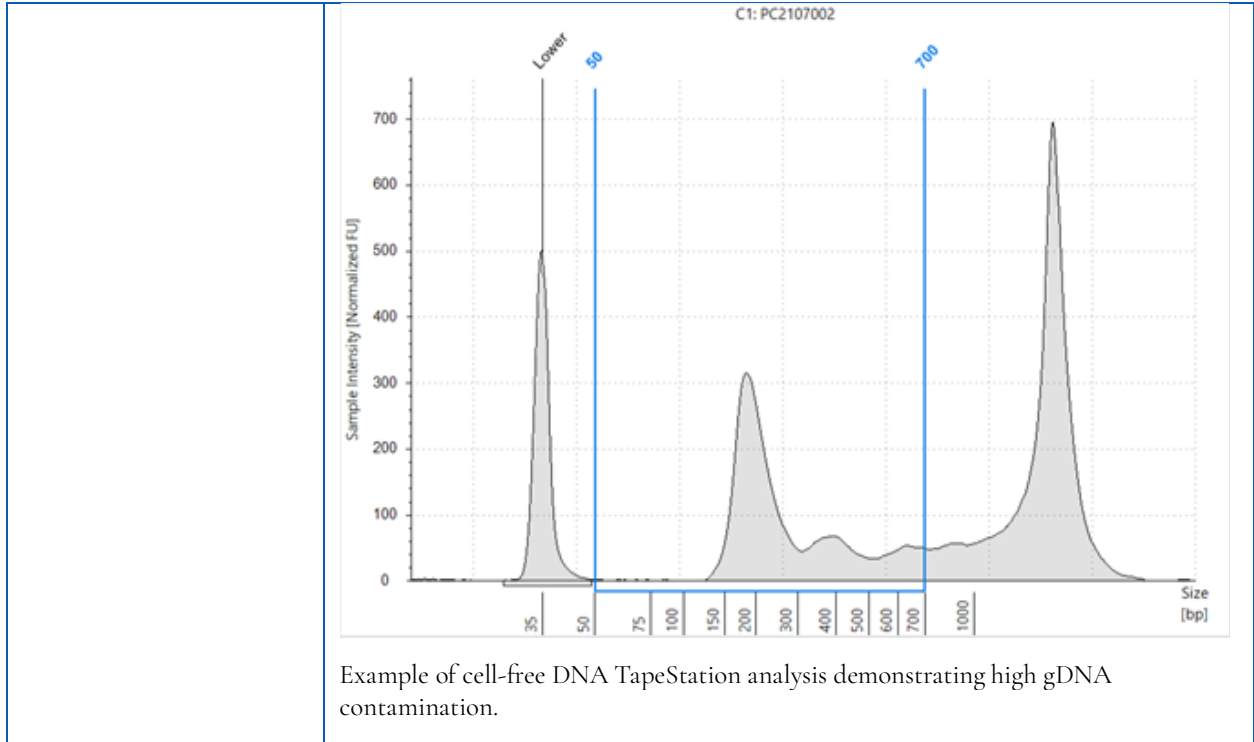
10. Appendix A: Index Sequences

i7 Index Sequences	
Index ID	**Index Barcode Sequence for NextSeq Sample Sheet**
Pi713	GAAACCCA
Pi714	TGTGACCA
Pi715	AGGGTCAA
Pi716	AGGAGTGG
Pi717	ACGTTACC
Pi718	CTGTGTTG
Pi719	TGAGGTGT
Pi720	GATCCATG
Pi721	GCCTATCA
Pi722	AACAACCG
Pi723	ACTCGTTG
Pi724	CCTATGGT
Pi725	TGTACACC
Pi726	GTATGCTG
Pi727	TGATGTCC
Pi728	GTCCTTCT
Pi729	ATAAGGCG
Pi730	CTTACCTG
Pi731	CGTTGCAA
Pi732	GATTCAGC
Pi733	TCACGTTC
Pi734	TGTGCGTT
Pi735	TAGTTGCG
Pi736	ACAGCTCA
Pi737	GTTAAGGC
Pi738	AAGCCACA
Pi739	ACACGGTT
Pi740	CAGCGATT
Pi741	TAGTGACC
Pi742	CGAGACTA
Pi743	GACATGGT
Pi744	GCATGTCT
Pi745	ACTCCATC
Pi746	CGAAGAAC
Pi747	GGTGTCTT
Pi748	AAGAAGGC
Pi749	CATGTTCC
Pi750	GTGCCATA

i7 Index Sequences	
Index ID	Index Barcode Sequence for NextSeq Sample Sheet
Pi751	CCTTGTAG
Pi752	GCTGGATT
Pi753	TAACGAGG
Pi754	ATGGTTGC
Pi755	CCTATACC
Pi756	TTAGGTCG
Pi757	GCAAGATC
Pi758	AGAGCCTT
Pi759	GCAATGGA
Pi760	CTGGAGTA

i5 Index Sequences	
Index ID	Index Barcode Sequence for NextSeq Sample Sheet
Pi513	TCGATTAG
Pi514	TGTTCTAG
Pi515	GGAACTTA
Pi516	TAGGTCTA
Pi517	TTGTCGGT
Pi518	TTGCCACT
Pi519	AGTCTGTG
Pi520	AAGTGTCG
Pi521	CACAAGTC
Pi522	AGTCTCAC
Pi523	CATGGAAC
Pi524	CTCAGCTA
Pi525	TTGCGAAG
Pi526	CATACCAC
Pi527	CTACAGTG
Pi528	TAGCGTCT
Pi529	TGGAGTTG
Pi530	AGCGTGTT
Pi531	ACCATCCA
Pi532	GCTTCGAA
Pi533	GTGGTGTT
Pi534	ACAGCTCA
Pi535	TTCCTGTG
Pi536	GGTTGTCA
Pi537	TCTCTAGG
Pi538	CGTTATGC
Pi539	AAGCACTG
Pi540	GAGATACG
Pi541	TCTTGACG
Pi542	GTTCATGG
Pi543	GAAGTACC
Pi544	ATAGCGGT
Pi545	ACCTGGAA
Pi546	AGGTTCGA
Pi547	TGGCACTA
Pi548	TTCGTACC
Pi549	CGATGCTT
Pi550	GTATTGGC
Pi551	GCATACAG
Pi552	GTCCTAAG
Pi553	TGGCATGT
Pi554	ACTCCATC
Pi555	ATCGATCG
Pi556	CTGGAGTA
Pi557	TGGTAGCT

i5 Index Sequences	
Index ID	**Index Barcode Sequence for NextSeq Sample Sheet**
Pi558	CTTGTCGA
Pi559	CGGTCATA
Pi560	AGTTGGCT