Leaders in Decision Medicine™

oncoReveal™ MGMT & MLH1 Methylation

USER MANUAL

UM-0076 version 2.0

For Research Use Only. Not for use in diagnostic procedures.

Revision History

Version 1.0	Initial release
Version 2.0	Updated consumable catalog numbers in section 4.4 Updated examples in section 9.3 Minor formatting and grammatical changes to improve consistency and clarity

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1. Product Introduction

The **oncoReveal™ MGMT & MLH1 Methylation** Panel targets specific genetic sequences in DNA that have undergone a bisulfite conversion for methylation analysis. This panel covers CpG sites within *MGMT* and *MLH1*. For more information on the panel specifications, see the oncoReveal™ MGMT & MLH1 Methylation Panel product sheet (*Doc. No. MK-0052*).

2. Product Description

The oncoReveal™ MGMT & MLH1 Methylation 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. Pairs of DNA oligos designed for each region of interest are used in the first round of gene-specific PCR (GS-PCR) and the products are subsequently purified via size selection. After purification, a second round of PCR adds index adaptors and P5 & P7 sequences to each library for sample tracking and sequencing. The final libraries are further purified and can be sequenced on the Illumina sequencing platform.

The panel requires an indexing kit, available in low or high throughput options, and the resulting libraries are designed for sequencing on the Illumina platform using a paired-end read length of 2×150. The workflow of this panel can be performed and loaded onto the sequencing instrument within one day. In addition, this protocol contains numerous stopping points for users who have time limitations.

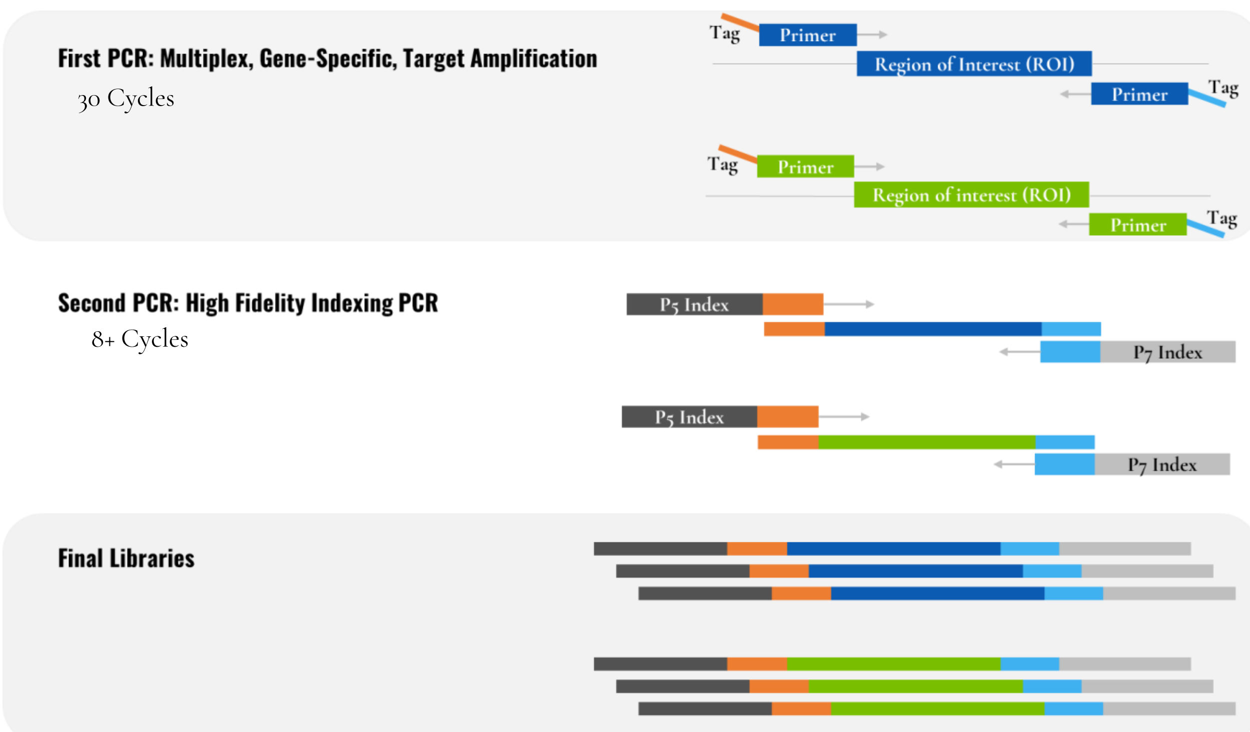


Figure 1. Overview of the oncoReveal™ MGMT & MLH1 Methylation Panel library preparation.

3. General Laboratory Guidelines

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

- **Work areas:** To reduce the risk of contamination from PCR amplicons, supplies should not be moved from one area to another. Separate storage areas (refrigerator, freezer) should also be designated for pre- and post-PCR products.

- **Lab cleanliness:** To further reduce the possibility of contamination, work areas should be cleaned between experiments with laboratory cleaning solution (70% alcohol or freshly-made 10% hypochlorite solution). A periodic cleaning of the floor is also recommended.

- **Floor:** Items that have fallen to the floor are assumed to be contaminated and should be discarded. If a sample tube or non-consumable item has fallen to the floor and remained sealed, the outer surface of the item should be thoroughly cleaned before use using 70% alcohol or freshly-made 10% hypochlorite solution. Gloves should be changed after handling a contaminated item.

- **Aliquot reagents:** Frozen reagents should be 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 cases 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™ MGMT & MLH1 Methylation Panel *Part No.: HDA-HR-1010-24*

Reagent	Use	Area Use	Storage
Gene-Specific PCR Master Mix (2x)	Gene-Specific PCR	Pre-PCR	-25°C to -15°C
oncoReveal™ MGMT & MLH1 Methylation Panel Oligo Pool	Gene-Specific PCR	Pre-PCR	-25°C to -15°C
Indexing PCR Master Mix (2x)	Indexing PCR	Pre-PCR	-25°C to -15°C

4.2 Indexing Kits

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

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

4.3 User-Supplied Reagents

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

[2] The 200 mM Tris-HCl, pH 7.0 reagent is only required for denaturing libraries for sequencing on the Illumina NextSeq or MiniSeq. This reagent is not needed if sequencing on the MiSeq.

4.4 Other Consumables

Compatible Sequencing Reagents

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

‡ *Indicates the flowcells the oncoReveal™ MGMT & MLH1 Methylation Panel has been validated on. However, libraries generated using this protocol are compatible with all Illumina sequencers.*

General Laboratory Consumables

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

Consumable	Area Use	Supplier
1.5 mL microcentrifuge tubes	Pre- and Post-PCR	General lab supplier
96-well PCR plates, 0.2 mL	Pre- and Post-PCR	Fisher Scientific, Cat# 14-222-334 or equivalent
Microplate sealing film	Pre- and Post-PCR	Fisher Scientific, Cat# 14-222-347 or equivalent
Conical tubes, 15 mL	Pre- and Post-PCR	General lab supplier
Conical tubes, 50 mL	Post-PCR	General lab supplier
Low retention, aerosol filter pipette tips	Pre- and Post-PCR	General lab supplier
Solution basin (trough or reservoir)	Pre- and Post-PCR	Fisher Scientific, Cat# 13-681-506 or equivalent
Qubit Assay tubes	Post-PCR	Invitrogen, Cat# Q32856

4.5 Equipment

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

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

5. Workflow

The following chart demonstrates the workflow for performing the oncoReveal™ MGMT & MLH1 Methylation Panel library preparation.

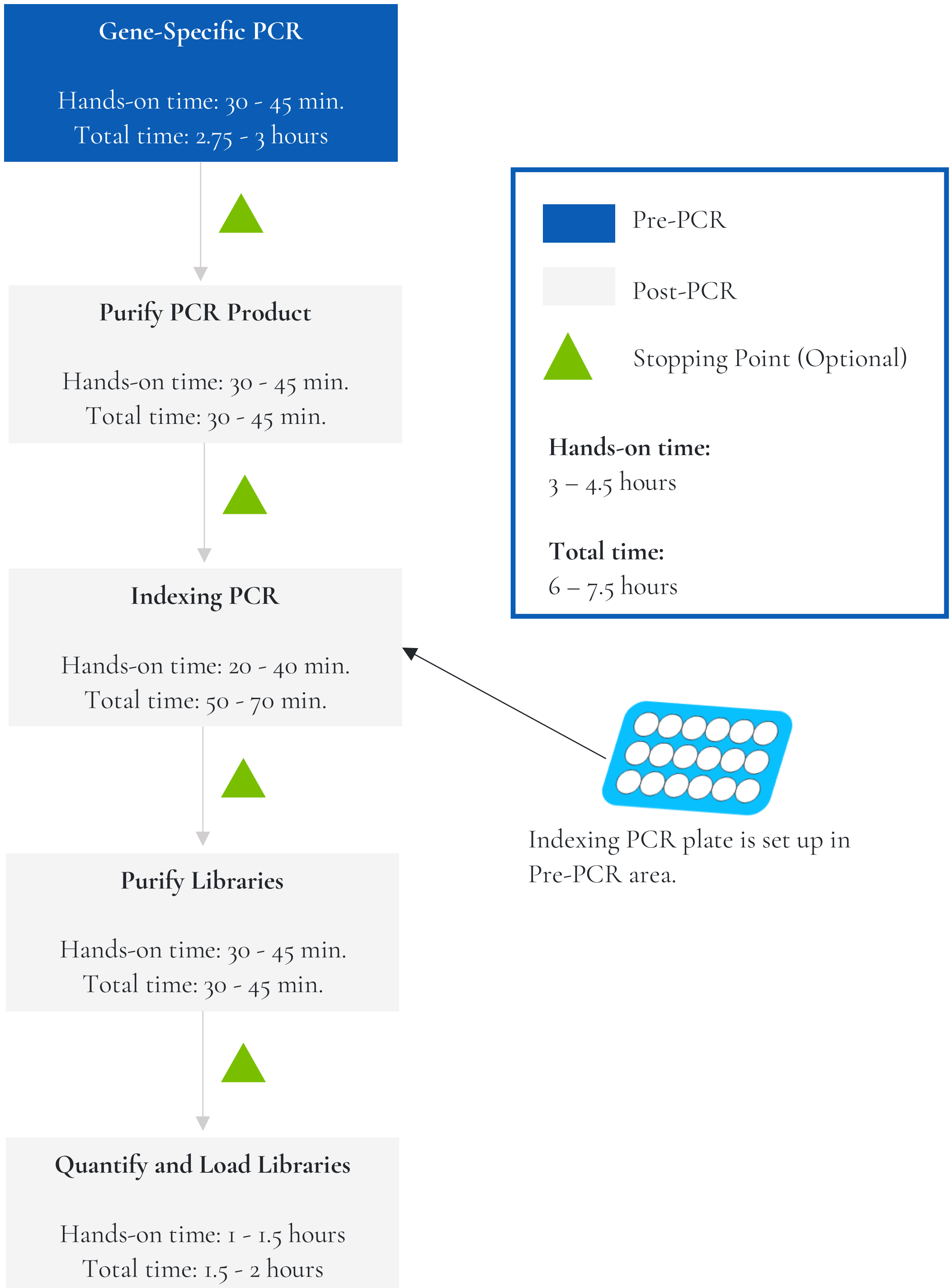


Figure 2. Library preparation workflow for oncoReveal™ MGMT & MLH1 Methylation Panel. The workflow can be completed within one day but contains multiple optional stopping points for users with time constraints.

Symbol	Description
	Information: Information that follows this symbol is important and may require action.
	Optional Stopping Point: A point in the workflow at which work may be safely paused, and samples can be stored appropriately.
	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. DNA Input Information

The following protocol includes information for preparing libraries using DNA that has undergone a bisulfite or enzymatic conversion.

The recommended DNA input is **5 – 20 ng** of bisulfite converted DNA per PCR reaction.

7. Library Preparation Protocol

Hands-on Time	3 – 4.5 hours
Total Time	6 – 7.5 hours

7.1 Gene-Specific PCR (GS-PCR) and Purification

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

Amplify Genomic DNA Targets

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

For this portion of the protocol prepare a pre-chilled benchtop cooler. The gene-specific PCR Master Mix (GS-PCR MMX) and the custom oligo pool should be kept in the cooler until needed.

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

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

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

GS-PCR Mix 1	
Reagent	**Volume (µL)**
Gene-Specific PCR Master Mix	12.5
oncoReveal™ MGMT & MLH1 Methylation Panel Oligo Pool	5.0
Subtotal	**17.5**

2. **Transfer:** Transfer **17.5 µL** of GS-PCR Mix 1 to each sample well in a PCR plate, strip tube, or PCR tube.

3. **Dilute input DNA:** Add **7.5 µL** of DNA (diluted if necessary)* to each sample well containing GS-PCR Mix 1. Add **7.5 µL** of nuclease-free water to the no-template control well.

Final GS-PCR Mix	
Reagent	**Volume (µL)**
GS-PCR Mix 1	17.5
DNA (or water)	7.5
Total	**25.0**

** 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.

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

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

GS-PCR Program		
Temperature	**Time**	**Number of Cycles**
95°C	15 min	1
98°C 56°C	1 min 1 min	6
95°C 66°C	30 sec 1 min	24
8°C	Hold	1

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

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

Gel Image After Gene-Specific PCR

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

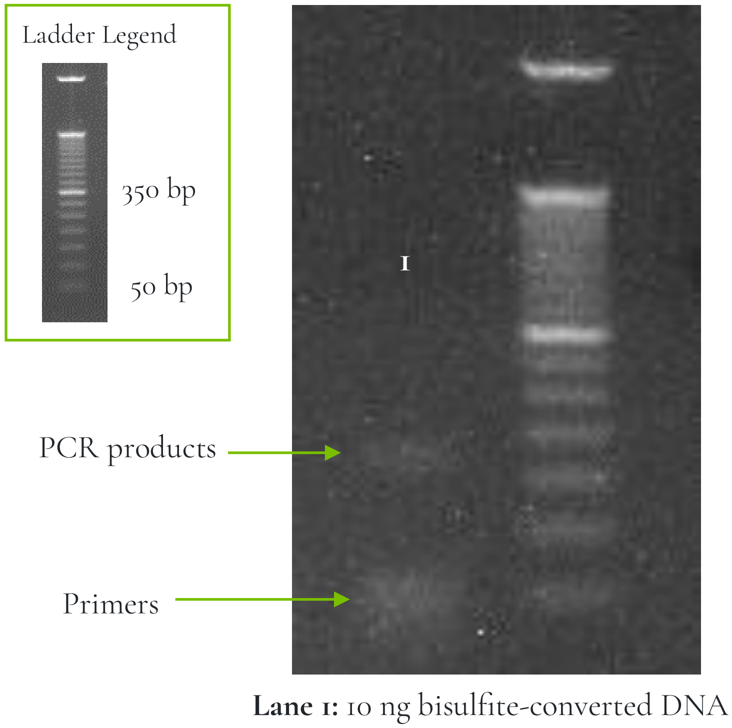


Figure 3. Analysis of GS-PCR product on 2% agarose gel. PCR product bands may appear faint when using low quantities of DNA.

Purify the Gene-Specific PCR Product

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

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

Before beginning the purification:

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

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

- If gene-specific PCR products were stored at -20°C, remove from the freezer to thaw at room temperature before 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 into 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 water to sample**: Add **25 µL** of nuclease-free water to each well containing GS-PCR product to bring the reaction volume to 50 µL.

4. **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.

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

6. **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.

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

8. **Wash beads:** With the PCR plate still on the magnetic rack add **150 µL** of freshly prepared 70% ethanol to each well without disturbing the beads. Incubate at room temperature for 30 seconds, and then remove the supernatant from each well.

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

9. **Second wash:** Repeat step 8 for a second 70% ethanol wash. Remove the supernatant from each well. The unused solution of ethanol can be used to purify the libraries after the indexing PCR is completed.

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

11. **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 the beads are flaky, they are over-dried. Beads that are too dry may be difficult to resuspend.

12. **Resuspend beads:** Remove the PCR plate from the magnetic rack and resuspend the dried beads in each well using **64 µL** of nuclease-free water. Gently pipette the suspension up and down 10 times. If bubbles form on the bottom of the wells, briefly centrifuge and mix again.

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

13. **Incubate and Elute:** Incubate for 5 minutes to fully elute the product.

14. **Separate GS-PCR product from beads:** Place the reactions on a magnetic rack at room temperature until the solution appears clear. Transfer **62 µL** of clear supernatant from each well to a new PCR plate.

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

7.2 Index PCR & Purification

Indexing PCR: Amplify the Libraries

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 pre-chilled cooler. The Indexing PCR Master Mix should be kept in the cooler until it is needed.

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

Indexing Primer Mix (per reaction)	
Reagent	**Volume (µL)**
P1700 Pillar Index	4.0
P1500 Pillar Index	4.0
Subtotal	**8.0**

2. **Prepare Indexing PCR Mix 1:** Briefly vortex and centrifuge the Indexing PCR Master Mix before use. Prepare Indexing PCR Mix 1 by combining Indexing PCR Master Mix and water with sufficient overage.

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

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

Indexing PCR Mix 2 (per reaction)	
Reagent	**Volume (µL)**
Indexing Primer Mix	8.0
Indexing PCR Mix 1	36.0
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 GS-PCR products were stored at -20°C after purification, ensure that they have been thawed at room temperature before proceeding.

4. **Add purified GS-PCR product:** Aliquot **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**

5. **Mix and spin:** Pulse vortex the sealed reactions on a medium setting for 5 – 10 seconds to mix. Briefly centrifuge the reactions to remove any bubbles.

6. **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 66°C 72°C	30 sec 30 sec 1 min	8*
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.

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

Before beginning the purification:

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

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

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

Library Purification

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 µL 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 for 5 minutes at room temperature.

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 to a new plate.

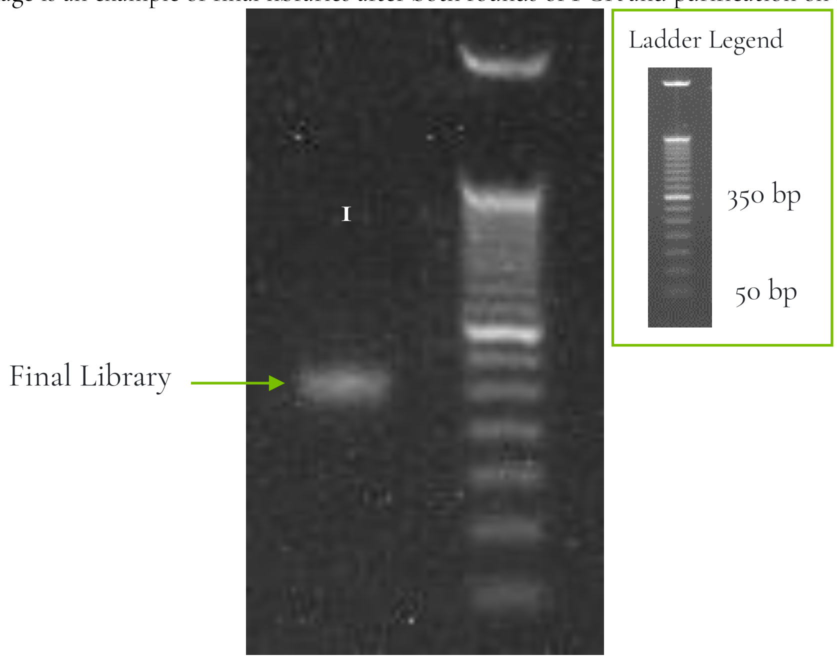
TIP: During the incubation and magnetic separation of the beads, cover the samples with microplate sealing film and prepare the solutions needed for quantitation performed in the next section.

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

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

Final Library Gel Image

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



Lane 1: 10 ng bisulfite-converted DNA

Figure 4. Gel analysis of final library material.

7.3 Quantitation and Normalization of Purified Libraries

Prior to sequencing libraries must be quantified, normalized, and then pooled together. The following section describes how to quantify libraries using the Qubit system. Other library quantification methods, such as qPCR quantification kits or TapeStation, 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.

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 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 of Qubit working solution and 1 µL library (1:200 dilution). Repeat steps 5–7.

8. **Calculate concentration**: 0.83 ng/µL of library is equal to **5 nM**. Example calculation is below. Adjust dilution factor accordingly.

   2 µL of library + 198 µL Qubit working solution:

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

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.

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

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

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

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

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.

4. **Quantify library pool (recommended):** The libraries prepared using the oncoReveal™ MGMT & MLH1 Methylation Panel cluster very efficiently on the MiSeq.

   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.

8. 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 them are the estimated throughput of the flowcell and sequencing platform, the desired sequencing depth, as well as the number of unique index combinations available.

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

Table 1. Sequencing recommendations.

Sequencing Configuration	Index	Recommended Coverage	Recommended Reads Per Sample
2 × 150	**Dual** (8 bp each)	Mean: 2000x Min: 400x	24,000 PE reads

Libraries generated with this protocol require 33% PhiX DNA to be added prior to sequencing.

Table 2. Multiplexing recommendations for Illumina sequencers.

Sequencing Flowcell	Estimated Instrument Output (million PE reads)	Estimated Maximum Sequencing Batch Size
MiSeq Nano	2	45
MiSeq Micro	8	180
MiSeq v2	30	675
MiSeq v3	50	>1,000

Calculations assume ≥ 80% effective on-target rate after read mapping and minimum segment coverage > 20% of the mean coverage.

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

For this portion of the protocol prepare a pre-chilled cooler.

Sequencing Using Illumina MiSeq™ (v2 or v3) Reagents

MiSeq recommended final library pool loading concentration: 15 pM
MiSeq recommended PhiX spike-in concentration: 12.5 pM

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

1. **Normalize:** Dilute libraries to 5 nM, as demonstrated in the previous section "*Quantitation and Normalization of Purified Libraries."*

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

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

3. **Denature the library pool:** Label a new 1.5 mL microtube for the denatured 25 pM library pool.

   a. Denature the 5 nM library pool by combining **5 µL** of the library pool and **5 µL** of the freshly prepared 0.2 N NaOH.
   b. Vortex thoroughly for 10 seconds and centrifuge for 1 minute.
   c. Let the solution stand at room temperature for 5 minutes.
   d. Add **990 µL** of Illumina's HT1 hybridization buffer to the denatured library pool.
   e. Invert the mixture several times, centrifuge briefly, and place on ice.

4. **Dilute library mix to 15 nM and add PhiX control:** Label a new 1.5 mL microtube for the 15 pM library mix. Combine 240 µL of the 25 pM library mix (step 2) with 120 µL of Illumina's HT1 solution and 240uL 12.5pM PhiX. 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.

5. **Load MiSeq cartridge:** Using a clean 1000 µL tip puncture the foil cap above the sample loading well on the MiSeq cartridge. Load the **600 µL** library pool and PhiX mixture into the cartridge, ensuring that the solution has reached the bottom of the well.

6. **Run the MiSeq:** Sequence the libraries on the MiSeq per the manufacturer's instructions using a paired-end read length of 150 (2×150) and two indexing reads of 8 cycles each. See *"MiSeq System User Guide" (part #15027617).*

7. **Store** diluted libraries and mixtures at -20°C for long-term storage.

Preparing a Sample Sheet for Sequencing

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

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

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

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

1. Open the Pillar sample sheet generator and enter user input in the shaded cells. Cells that are shaded blue are required and cells that are shaded grey are optional.

2. Enter the "Sample_ID" for each sample. Each Sample_ID must be unique and contain only alphanumeric characters, dashes (-), and underscores (_). All other characters are not allowed. To check that the Sample_ID meets all requirements click "Reset Sample_ID color" and then click "Check Sample_ID".

3. If text is green, the Sample_ID is acceptable. If text is red, Sample_ID is not acceptable. Change Sample_ID accordingly and repeat step 2 until all text is green.

4. Next, enter indices into appropriate fields. Index sequences will be populated once the index_ID is entered.

5. Check that the index combination for each sample is unique. If "Check_index_uniqueness" column is green, then all index combinations are unique. If the column is red for a sample, then the index combination is not unique. Do not load samples together in the same run that have the same index combination.

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

9. Methylation Calling with PiVAT®

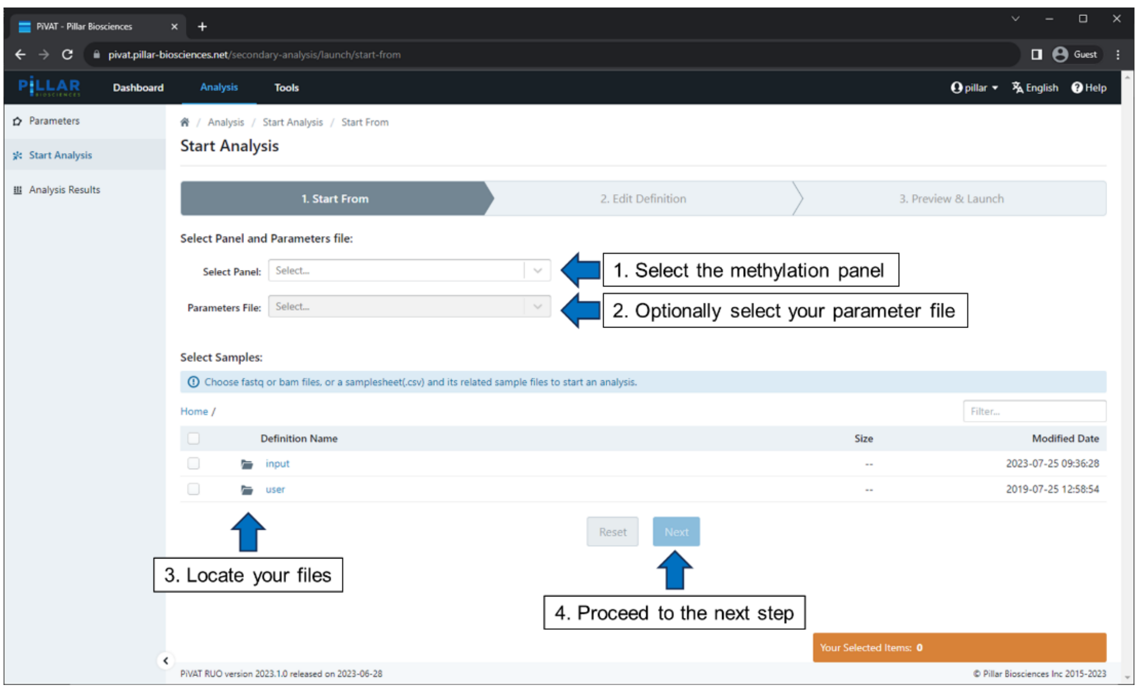
The oncoReveal™ MGMT & MLH1 Methylation Panel data analysis is performed using the methylation module in Pillar's secondary analysis pipeline, PiVAT®. This module accurately identifies methylation levels at targeted CpG sites covered by the oncoReveal™ MGMT & MLH1 Methylation Panel. The output of the methylation analysis is provided in a Microsoft Excel file format, allowing flexibility in post-analysis data reporting. For example, final methylation values can be adjusted in the Excel output file based on the expected tumor content of the sequenced samples.

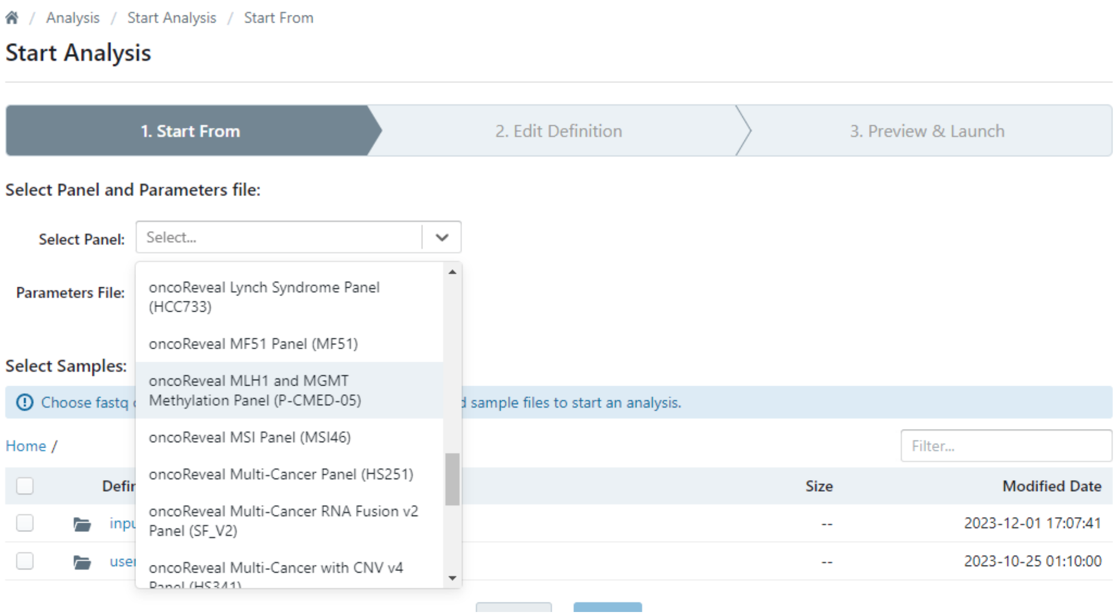
The following section provides a walkthrough explaining how to initialize the analysis and how to understand the output format generated by PiVAT.

9.1 Starting your PiVAT Run

Identify negative control samples in PiVAT using the following steps:

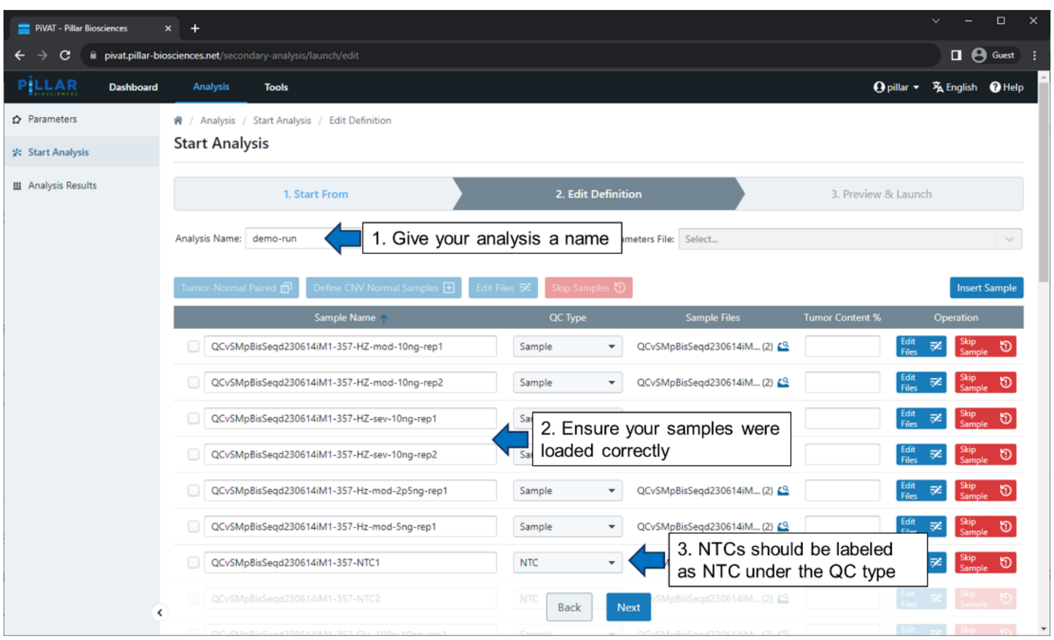
1. Set up the analysis run by selecting the correct panel and FASTQ output.



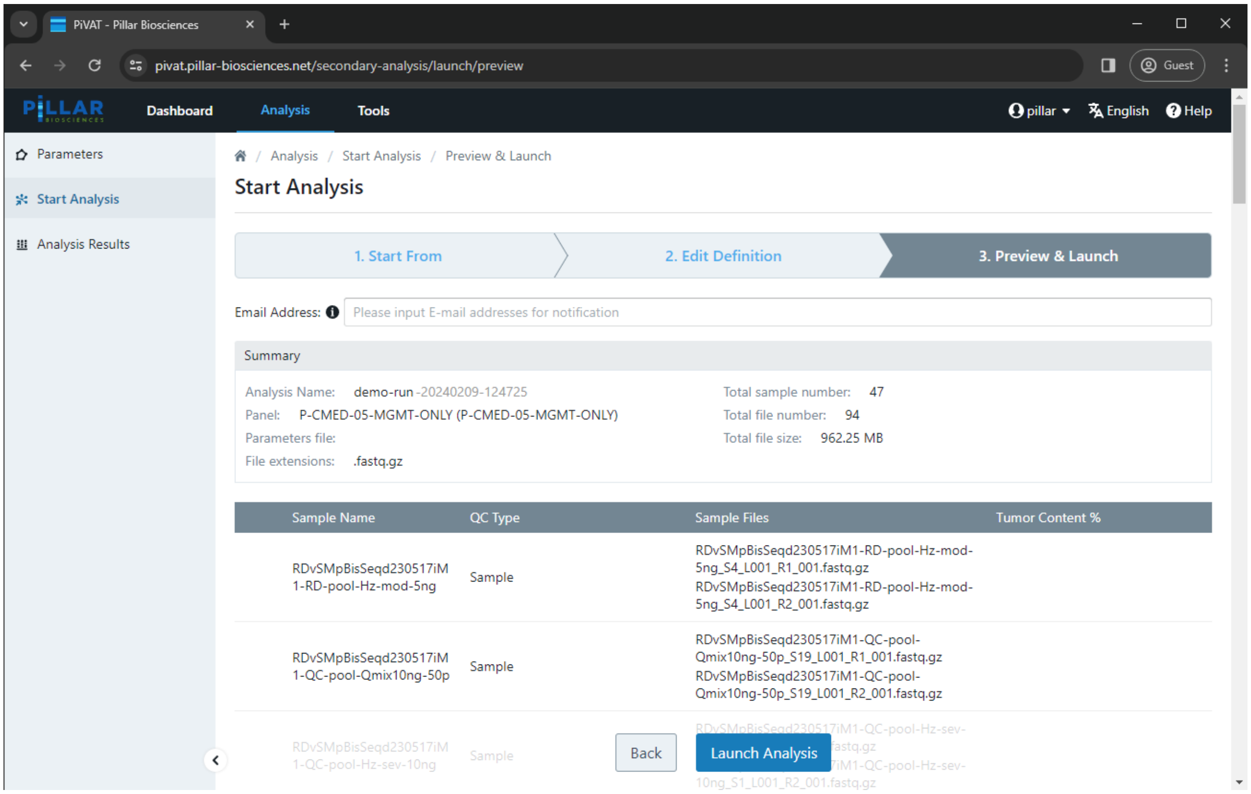


The name of this panel in PiVAT is "oncoReveal™ MLH1 and MGMT Methylation Panel (P-MED-05)"

2. Input an appropriate name for the analysis run and verify that the samples have been input correctly.



3. Verify the run setup. Remember to scroll all the way down to confirm that the negative control samples are reflected appropriately.



4. Once the run setup is confirmed, launch the analysis.

9.2 Output Format in PiVAT

Important: PiVAT reports methylation levels as percentages. It is recommended that users make inferences about differences in methylation levels based on existing literature and on their own prior testing and controls.

The output from PiVAT will be exported in a Microsoft Excel file containing the following information:

1. The methylation levels, computed at 3 levels: per-site, per-amplicon, and per-gene
2. Bisulfite conversion rates
3. Overall sequencing and mapping statistics
4. Run-specific parameters

The data contained in the Excel file is organized in multiple sheets:

Sheet Name in Excel Output File	Description
Methylation Summary By Sample	High-level summary sheet that contains samples in each row and methylation summary by gene and amplicon as columns. The methylation summary consists of mean, standard deviation, per-amplicon coverage, and coefficient of variation as a percentage.
Methylation Site Type	Methylation and bisulfite conversion levels at each of the targeted positions across each sample.
Methylation Summary By Site	A variation of the "Methylation Site Type" sheet, wherein the table is transformed to display CpG sites as rows and samples as columns, to make it easy to compare per-site methylation levels between samples.
Bisulfite Conversion Rates	A two-column table with each sample and its corresponding bisulfite conversion values (expressed as a percentage).
Overall Stats	Overall sequencing and mapping statistics. For a more detailed description of this sheet, see the *PiVAT® User Manual (Doc. No. UM-0073).*
Segment Coverage	Per-amplicon coverage (rows) across all samples (columns). Mean, standard deviation, and minimum coverages are reported.
Run Parameters	Parameters used within the PiVAT run, recorded for audit trail purposes.

9.3 Example PiVAT Output

Methylation Summary By Sample

Sample_ID	SampleType	Methylation_Mean:MGMT	Methylation_Mean:MLH1	Coverage_Mean: MM01.MLH1-Prm1.deg	Coverage_Mean: MM02.MLH1-Prm1	Coverage_Mean: MM03.MGMT-Prm1.deg	Coverage_Mean: MM04.MGMT-Ex1.deg	Coverage_Mean: MM05.MGMT-Int01.deg	Coverage_Mean: MM06.MGMT-pDMR1.deg
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	1.07	1.41	6692.62	13896.8	5433.23	3777.83	6174.2	14724.9
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep1	Sample	1.13	1.48	10856.3	21470	7887	4900.25	9358.1	17377.7
QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep2	Sample	53.04	51.24	7082.25	12544.2	4811.85	4169.83	8589	12504.6
QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep1	Sample	53.2	53.39	7442.88	14051	5464.54	3633.33	8642.3	10779.5
QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep1	Sample	52.26	57.17	5289.62	11522.6	8114.23	7355.08	9847.8	16644.1
QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep2	Sample	52.24	55.03	5463.62	9999.8	8263.77	7064.75	9465	15836.5
QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep2	Sample	92.09	90.29	9778.88	16392.4	5219.92	4150.08	9808.6	12111.6
QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep1	Sample	93.3	90.59	8618.25	16680.8	5414.69	3748.42	9730.5	8900.87
QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep2	Sample	96.56	97.23	7970.75	13553	6919.69	5673.67	11519.6	10990.9
QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep1	Sample	96.03	97.14	8093.25	14395	6595.23	5817.25	9417.3	10114.3
QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep2	Sample	70.36	69.52	5043.38	14415.8	7971.46	8177	5973.2	9792.87

Sample_ID	Methylation_Mean:MM01.MLH1-Prm1.deg	Methylation_Mean:MM02.MLH1-Prm1	Methylation_Mean:MM03.MGMT-Prm1.deg	Methylation_Mean:MM04.MGMT-Ex1.deg	Methylation_Mean:MM05.MGMT-Int01.deg	Methylation_Mean:MM06.MGMT-pDMR1.deg	STDEV:MM01.MLH1-Prm1.deg	STDEV:MM02.MLH1-Prm1	STDEV:MM03.MGMT-Prm1.deg	STDEV:MM04.MGMT-Ex1.deg	STDEV:MM05.MGMT-Int01.deg	STDEV:MM06.MGMT-pDMR1.deg	%CV:MM02.MLH1-Prm1	%CV:M 01.MI Prm1.
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	1.51	1.24	0.97	1.03	0.82	1.35	0.61	0.43	0.85	1.09	0.68	0.73		
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep1	1.49	1.46	1.08	0.94	1.13	1.32	0.6	0.57	0.69	0.55	0.92	0.52		
QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep2	50.03	53.18	52.92	54.69	60.47	46.86	2.33	3.83	1.58	2.6	3.01	4.51	0.07	
QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep1	52.53	54.77	55.31	59.35	57.64	43.5	2.34	2.95	1.35	3.9	2.47	4.29	0.05	
QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep1	55.34	60.11	53.56	55.26	60.56	43.21	0.69	0.71	0.42	0.58	0.45	0.72	0.01	
QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep2	54.88	55.27	53.16	55.69	59.74	43.68	1.11	0.53	0.37	0.76	1.07	0.61	0.01	
QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep2	88.04	93.89	96.8	91.53	95.81	85.98	3.71	5.31	2.7	5.89	3.3	11.77	0.06	
QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep1	90.15	91.29	96.94	91.64	94.24	90.84	2.94	7.31	2.75	5.69	5.26	7.69	0.08	
QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep2	98.11	95.82	97.07	96.16	96.29	96.61	1.56	0.73	1.09	1.22	1.47	1.41	0.01	
QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep1	98.15	95.52	96.37	96.46	95.61	95.68	1.44	0.87	1.01	1.3	1.54	1.75	0.01	
QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep2	71.02	67.1	76.16	73.4	77.96	57.84	0.59	5.55	5.91	0.55	9.31	1.25	0.08	

NOTE: In the provided example, the data in some columns have been removed for brevity and for visual clarity.

Column Label	Description
Sample_ID	Sample name
SampleType	Sample type (e.g., sample, positive control, or no-template control)
Methylation_Mean	Mean methylation level *Gene-specific methylation mean is represented in Column C in the example above.* *Amplicon-specific methylation mean is represented in Column G in the example above.*
Coverage_Mean	Mean amplicon coverage
STDEV	Standard deviation of the mean
%CV	Coefficient of specification for a specific amplicon **This column will be empty if the mean methylation level is below 5%.**

Methylation Site Type

Site-by-site methylation information for each sample.

Sample_ID	SampleType	Chromosome	Position	REF_(Unmethylated)	ALT	Location	Amplicon_ID	Gene_Symbol	Methylation_Rate_(%)
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034598	T	C	chr3:3703	MM02.ML	MLH1	1.439574
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034610	T	C	chr3:3703	MM02.ML	MLH1	1.755017
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034638	T	C	chr3:3703	MM02.ML	MLH1	1.201439
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034654	T	C	chr3:3703	MM02.ML	MLH1	1.230039
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034661	T	C	chr3:3703	MM02.ML	MLH1	0.583321
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034770	T	C	chr3:3703	MM01.ML	MLH1	1.165223
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034777	T	C	chr3:3703	MM01.ML	MLH1	1.194922
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034787	T	C	chr3:3703	MM01.ML	MLH1	1.43412
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034789	T	C	chr3:3703	MM01.ML	MLH1	0.896459
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034795	T	C	chr3:3703	MM01.ML	MLH1	1.330145
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034814	T	C	chr3:3703	MM01.ML	MLH1	1.885099
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034825	T	C	chr3:3703	MM01.ML	MLH1	2.852875
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr3	37034840	T	C	chr3:3703	MM01.ML	MLH1	1.314414
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	2.631579
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	1.343146
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	0.367985
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	1.325479
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	0.276091

Sample_ID	Methylated_Base_Coverage	Methylated+Unmethylated_Total_Count	Methylated_Base_Quality	Methylated_Base_Direction_Ratio	Consequence	Gene_ID	Feature	All_Features	HGVSC	Exon	Intron	Strand	Repeat	Methylation_Site_Type
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	200	13893	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-441			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	244	13903	38	-0.0F/R	upstream	4292	NM_0002	NM_0002	c.-429			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	167	13900	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-401			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	171	13902	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-385			1	8T	CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	81	13886	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-378			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	78	6694	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-269			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	80	6695	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-262			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	96	6694	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-252			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	60	6693	38	-0.02F/R	upstream	4292	NM_0002	NM_0002	c.-250			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	89	6691	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-244			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	126	6684	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-225			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	191	6695	38	0.0F/R	upstream	4292	NM_0002	NM_0002	c.-214			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	88	6695	38	0.01F/R	upstream	4292	NM_0002	NM_0002	c.-199			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	143	5434	38	0.0F/R	upstream	4255	NM_00241	NM_00241	c.-572			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	73	5435	38	0.01F/R	upstream	4255	NM_00241	NM_00241	c.-568			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	20	5435	38	0.0F/R	upstream	4255	NM_00241	NM_00241	c.-565			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	72	5432	38	0.0F/R	upstream	4255	NM_00241	NM_00241	c.-558			1		CpG Site
QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	15	5433	38	0.0F/R	upstream	4255	NM_00241	NM_00241	c.-551			1	5T	CpG Site

Methylation Summary By Site

An alternate view of the data also shown in the previous sheet, Methylation Site Type.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	Chromosome	Position	REF_(Unmethylated)	ALT	Location	Amplicon_ID	Gene_Symbol	Consequence	Gene_ID	Feature	All_Features	HGVSC	Exon	Intron	Strand	Repeat
2	chr3	37034598	T	C	chr3:3703	MM02.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-441			1	
3	chr3	37034610	T	C	chr3:3703	MM02.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-429			1	
4	chr3	37034638	T	C	chr3:3703	MM02.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-401			1	
5	chr3	37034654	T	C	chr3:3703	MM02.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-385			1	8T
6	chr3	37034661	T	C	chr3:3703	MM02.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-378			1	
7	chr3	37034770	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-269			1	
8	chr3	37034777	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-262			1	
9	chr3	37034787	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-252			1	
10	chr3	37034789	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-250			1	
11	chr3	37034795	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-244			1	
12	chr3	37034814	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-225			1	
13	chr3	37034825	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-214			1	
14	chr3	37034840	T	C	chr3:3703	MM01.ML	MLH1	upstream	4292	NM_0002	NM_0002	c.-199			1	
15	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	upstream	4255	NM_0024	NM_0024	c.-572			1	
16	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	upstream	4255	NM_0024	NM_0024	c.-568			1	
17	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	upstream	4255	NM_0024	NM_0024	c.-565			1	
18	chr10	1.31E+08	T	C	chr10:131	MM03.MG	MGMT	upstream	4255	NM_0024	NM_0024	c.-558			1	

	Q	R	S	T	U	V	W	X	Y
1	QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep1	QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep1	QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep2	QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep1	QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep2	QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep1	QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep2	QCvSBisSe31103QCPoHz-srep
2	1.201397	1.439574	83.57314	89.3086	52.34503	48.84815	63.1767	59.62405	62.2
3	1.448263	1.755017	92.76805	95.17506	55.18395	54.3882	76.81539	70.9183	78.2
4	2.440387	1.201439	83.73891	87.40239	51.20615	49.3902	67.40723	62.68729	70.
5	0.973179	1.230039	98.14193	98.38975	57.0158	56.98573	75.40661	70.61598	77.9
6	1.230597	0.583321	98.20743	99.16433	58.08886	56.27243	76.36017	71.66944	76.8
7	1.059517	1.165223	88.65166	87.28779	50.66505	49.74583	76.2223	70.71768	77.3
8	0.626439	1.194922	87.71177	85.11247	51.38366	48.17848	76.16115	71.41158	77.4
9	1.427387	1.43412	88.35132	85.84867	51.06812	48.61621	76.31024	72.07136	76.0
10	2.312298	0.896459	89.23309	86.06197	51.46427	48.84213	75.6771	71.44557	75.5
11	1.068435	1.330145	88.05849	85.02045	52.02902	48.0514	75.05717	70.58707	77.3
12	1.501336	1.885099	91.57479	88.38089	52.82258	50.05648	74.64367	70.94983	72.0
13	2.321297	2.852875	91.07889	90.70267	52.81473	51.78597	74.74055	70.22205	76.8
14	1.602653	1.314414	96.54212	95.9407	57.97958	54.94847	75.54109	70.7854	73.5
15	0.875079	2.631579	99.11374	99.27203	55.76501	54.37357	76.24541	77.40843	87.7
16	0.507228	1.343146	99.31659	98.81203	56.67764	52.98275	76.49141	76.62484	88.1
17	1.331136	0.367985	89.22963	88.77395	53.00037	50.46768	73.97404	77.79729	84.9
18	1.052632	1.325479	97.37717	95.9387	54.40073	52.93995	73.13746	78.19049	84.2

Bisulfite Conversion Rates

The bisulfite conversion rate as a percentage. The example shown is the bisulfite conversion rate across multiple samples.

	A	B
1	Sample_ID	Bisulfite_Conversion_Rate_(%)
2	QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	99.08861
3	QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep2	99.05102
4	QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep1	98.73202
5	QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep1	99.3224
6	QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep2	99.06695
7	QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep2	98.32635
8	QCvSMpBisSeqd231103iM5-QCPool1-Hz-sev-rep1	99.01107
9	QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep1	99.05223
10	QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep1	99.05187
11	QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep2	99.3119
12	QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep1	98.98893

Overall Stats

Detailed sequencing and mapping results from the run analyzed.

Stat	Sample: QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Sample: QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep2	Sample: QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep1	Sample: QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep1	Sample: QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep2	Sample: QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep2	Sample: QCvSMpBisSeqd231103iM5-QCPool1-Hz-sev-rep1	Sample: QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep1	Sample: QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep1	Sample: QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep2	Sample: QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep1
Total Reads	104566	123704	105650	125308	109974	124934	130728	119070	112462	119556	146518
Overall:Q=30	98.56	98.35	98.42	98.44	98.39	98.39	98.51	98.47	98.58	98.37	98.59
Overall:Q=20	99.61	99.57	99.58	99.59	99.58	99.58	99.62	99.61	99.63	99.57	99.61
Properly Paired Reads	102186	113886	100716	118204	103400	115726	123026	109542	106046	112850	144564
Properly Paired Read (%)	97.72	92.06	95.33	94.33	94.02	92.63	94.11	92	94.29	94.39	98.67
Mapped Reads	103868	114150	101534	118696	104311	116710	124584	109931	107291	113328	146059
Mapping Rate (%)	99.33	92.28	96.1	94.72	94.85	93.42	95.3	92.32	95.4	94.79	99.69
On Target Reads	103661	114124	101384	118647	104216	116610	124441	109847	107250	113285	145847
On Target Rate (%)	99.8	99.98	99.85	99.96	99.91	99.91	99.89	99.92	99.96	99.96	99.85
Insert Size Mean	129	129	129	129	125	129	123	128	124	129	129
Insert Size Median	131	131	131	131	122	131	122	131	122	131	131
Insert Size Std Dev	12	14	13	14	14	13	14	14	13	14	12
Stat	**:31103iM5**	**231103iM**	**31103iM5-**	**231103iM**	**231103iM**	**1103iM5-d**	**231103iM**	**231103iM**	**231103iM**	**231103iM**	**31103iM5**
Coverage_Mean	8641	9664	8544	9998	8406	9824	9795	9189	8510	9555	12201
STDEV	4195	2644	3202	3737	3019	3859	3938	2644	3752	3377	5442
Coverage_Median	6695	10992	8645	9850	7973	9810	9000	9418	7628	9467	10858
Coverage_Max	14729	13557	14055	16651	14420	16396	17347	14397	16791	15843	21476
Coverage_Min	3778	5673	3633	5289	5043	4148	5754	5816	5358	5462	4900
Total_Number_Of_Reads	104126	114252	101650	118804	104477	117056	124734	110012	107735	113439	146399
Total_Valid_Reads	101419	113250	100038	117560	102752	114918	122087	108856	105416	112193	143721
On_Target_Ratio	0.974003	0.99123	0.984142	0.989529	0.983489	0.981735	0.978779	0.989492	0.978475	0.989016	0.981708

Segment Coverage

Amplicon mean coverage, standard deviation, and minimum coverage across all samples.

Target_Name	Region	Segment_Size	GC_Content_(%)	Coverage_Mean:QCvSMpBisSeqd231103iM5-QCPool1-0p-control-rep2	Coverage_Mean:QCvSMpBisSeqd231103iM5-QCPool1-zym-ref-rep2	Coverage_Mean:QCvSMpBisSeqd231103iM5-QCPool1-50p-control-rep1	Coverage_Mean:QCvSMpBisSeqd231103iM5-QCPool1-Qia-mix-rep1	Coverage_Mean:QCvSMpBisSeqd231103iM5-QCPool1-Hz-mod-rep2	Coverage_Mean:QCvSMpBisSeqd231103iM5-QCPool1-100p-control-rep2	Coverage_Mean:QCvSMpBisSeqd231103iM5-QCPool1-Hz-sev-rep1
MM02.MLH1-Prm1_ROI	chr3:3703	80	32.5	13902	13556	14055	11527	14419	16395	17346
MM01.MLH1-Prm1.deg_ROI	chr3:3703	92	28.78788	6696	7974	7445	5291	5045	9782	6072
MM03.MGMT-Prm1.deg_ROI	chr10:131	83	31.70732	5434	6920	5465	8115	7972	5221	8999
MM06.MGMT-pDMR1.deg_ROI	chr10:131	98	42.44604	14728	10993	10783	16650	9797	12116	10439
MM04.MGMT-Ex1.deg_ROI	chr10:131	64	33.33333	3779	5675	3634	7357	8180	4151	12448
MM05.MGMT-Int01.deg_ROI	chr10:131	106	34.45946	6176	11521	8646	9850	5975	9810	5756

10. Troubleshooting Library Preparation

Low Yield of Gene-Specific Product

Potential Cause	Solution
DNA quantity or quality	The recommended input for the assay is 5 – 20 ng of bisulfite converted 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 5 – 20 ng of bisulfite converted 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.

Potential Cause	Solution
	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 (or 1 nM if sequencing on the MiniSeq) library mix using Qubit or RT-PCR. Dilute the denatured library mix as needed to adjust for the difference in concentration.
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.

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

11. Appendix A: Index Sequences

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

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

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