Bisulfite methylation library production and analysis using the Ion AmpliSeq™ Library Kit Plus

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The bisulfite method is the most commonly used technique for identifying specific methylation patterns of a DNA sample. This protocol combines a bisulfite kit protocol with the Ion AmpliSeq™ Library Kit Plus protocol to create (NGS) libraries for target DNA methylation assessment during sequencing. DNA is treated with bisulfite, which converts unmethylated cytosines to uracil but does not change methylated cytosines. Next, the Ion AmpliSeq™ Library Kit Plus and Ion AmpliSeq™ panels are used to prepare amplicon libraries of the bisulfite-treated DNA for sequencing on Ion Torrent™ sequencers. The methylation_analysis plugin performs alignment and methylation calling for amplicons on both the Watson (W) and Crick (C) strands. Each amplicon can have zero, one or more designated CpG targets (hotspots) of interest. A summary report shows each barcode name along with the sample name, the total number of reads covering the target CpGs, and the percentage of those reads that are methylated.

For more information about the bisulfite conversion reaction, see the *MethylCode™ Bisulfite Conversion Kit User Guide* (Pub. No. 25-1015).

For more information about the Ion AmpliSeq™ Library Kit Plus, see the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Required kits

Ion AmpliSeq™ Library Kit Plus

The Ion AmpliSeq™ Library Kit Plus (Cat. No. 4488990) provides reagents for manually preparing 24 libraries for 1- or 2-pool panels (16 libraries for 3-pool panels, and 12 libraries for 4-pool panels).

Component	Amount	Storage
	Cat. No. 4488990 (24 reactions)	
5X Ion AmpliSeq™ HiFi Mix (red cap)	120 µL	–30°C to –10°C
FuPa Reagent (brown cap)	48 µL	
Switch Solution (yellow cap)	96 µL	
DNA Ligase (blue cap)	48 µL	
25X Library Amp Primers (pink cap)	48 µL	
1X Library Amp Mix (black cap)	1.2 mL	
Low TE	6 mL	15°C to 30°C[1]

[1] Can be stored at –30°C to –10°C.

MethylCode™ Bisulfite Conversion Kit

The MethylCode™ Bisulfite Conversion Kit (Cat. No. MECOV50) provides reagents for 50 reactions. Store components at room temperature.

Component	Amount
CT Conversion Reagent	5 tubes
Dilution Buffer	1.5 mL
Resuspension Buffer	500 µL
Binding Buffer	30 mL
Wash Buffer	6 mL
Desulphonation Buffer	10 mL
Elution Buffer	1 mL
Spin Columns	50 columns
Collection tubes	50 tubes

Order bisulfite panels—Ion AmpliSeq™ Methylation Panel for Cancer Research

The Ion AmpliSeq™ Methylation Panel for Cancer Research enables the identification of methylation patterns of clinically relevant targets in a DNA sample, including targets associated with colon cancer, prostate cancer, Leukemia, and lymphoma.

The panel is a single pool (5X) with 40 amplicons. The amplicon size is 125–175 bp.

High-quality FFPE samples can be used with the panel.

1. Sign in to your account **AmpliSeq.com**.
2. In the **Ion AmpliSeq** pane, select the **Made-to-Order Panels** tab, then click the **Pre-designed by our community** button.
3. Select Ion AmpliSeq™ Methylation Panel for Cancer Research from the list of available community panels.
   Searching the list by *methylation panel* displays the Ion AmpliSeq™ Methylation Panel for Cancer Research.

Order bisulfite panels—custom panels

Bisulfite panels provide pools of primers for the amplification of target regions. The primers contain proprietary modifications that enable removal of primer sequences during library preparation for efficient target assessment during sequencing. Each panel includes one or more pre-made pools of primer pairs at 2X or 5X concentration for use with the Ion AmpliSeq™ Library Kit Plus.

1. Contact your sales representative.
2. Your sales representative connects you with the bisulfite white-glove service team.
   The bisulfite white-glove service team designs and uploads the panel to your account on the Ion AmpliSeq Designer website.
3. Sign in to your account on **AmpliSeq.com**, then order the amplification panel.

Required materials not supplied

In addition to the MethylCode™ Bisulfite Conversion Kit, the Ion AmpliSeq™ Library Kit Plus, and bisulfite panel, you need the following materials and equipment. Unless otherwise indicated, all materials are available through **thermofisher.com**.

Item	Source
Unmethylated Lambda DNA	Promega D1521
One of the following, or equivalent: • SimpliAmp™ Thermal Cycler • Applied Biosystems™ 2720 Thermal Cycler • Veriti™ 96-Well Thermal Cycler • ProFlex™ 96-well PCR System • GeneAmp™ PCR System 9700[1] or Dual 96-well Thermal Cycler	Various

Item	Source
Qubit™ 4 Fluorometer[2] and the Qubit™ dsDNA HS Assay Kit (DNA)	Q33226 Q32851
One of the following: • Ion Library TaqMan® Quantitation Kit and real-time PCR instrument • Agilent™ 2100 Bioanalyzer™ and Agilent™ High Sensitivity DNA Kit	4468802 Agilent G2939AA, 5067-4626
One of the following: • IonCode™ Barcode Adapters 1–384 Kit • Ion Xpress™ Barcode Adapters Kit • Ion Torrent™ Dual Barcode Kit 1–96	A29751 Various A39360
CpG Methylated Jurkat Genomic DNA, or equivalent quality methylated or unmethylated human gDNA control	SD1121
Ion 520™ & Ion 530™ ExT Kit – Chef	A30670
Ion 520™ Chip Kit or Ion 530™ Chip Kit	A27762 A27764
MicroAmp™ Optical 96-Well Reaction Plate with Barcode	N8010560, 4306737
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Film Compression Pad	4312639
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL	13-698-791 (**fisherscientific.com**)
Agencourt™ AMPure™ XP Kit	NC9959336, NC9933872 (**fisherscientific.com**)
DynaMag™–96 Side Magnet, or other plate magnet	12331D
Nuclease-free Water	AM9932
Ethanol, Absolute, Molecular Biology Grade	BP2818500 (**fisherscientific.com**)
Pipettors, 2–200 µL, and low-retention filtered pipette tips	(**fisherscientific.com**)

[1] Supported but no longer available for purchase.
[2] Qubit™ 2.0 Fluorometer and later are supported.

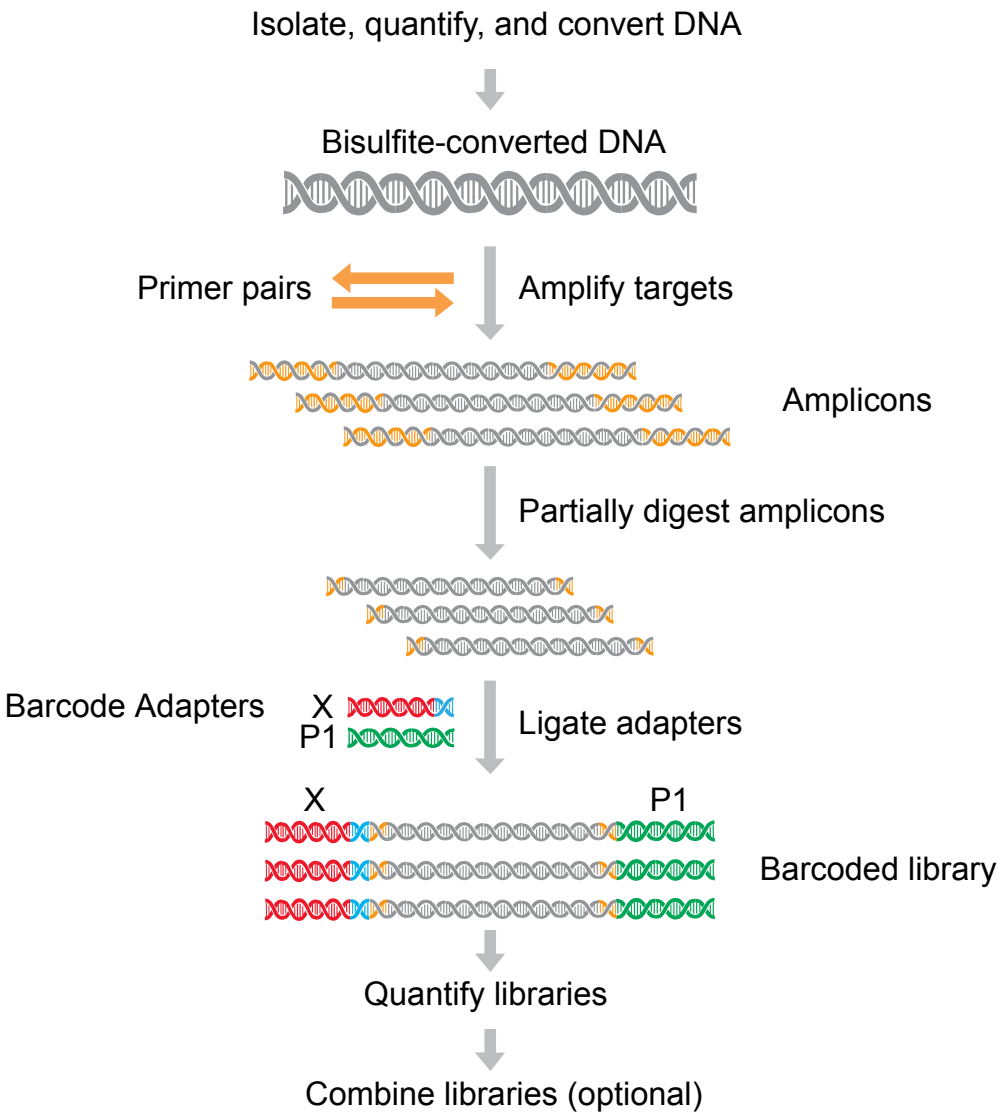
Recommended materials and equipment

Unless otherwise indicated, all materials are available through **thermofisher.com**. MLS: Fisher Scientific (**fisherscientific.com**) or other major laboratory supplier.

Item	Source
Qubit™ ssDNA Assay Kit	Q10212
Additional equipment	
Real-time PCR instrument (e.g., Applied Biosystems™ 7900HT, 7500, StepOne™, StepOnePlus™, ViiA™ 7, QuantStudio™ 3, QuantStudio™ 5, QuantStudio™7 , or QuantStudio™ 12K Flex Real–Time PCR Systems)	Various
Fisher Scientific™ Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge	14-100-143 (fisherscientific.com)
MicroAmp™ Adhesive Film Applicator	4333183
Nucleic acid isolation	
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069
MagMAX™ FFPE Total Nucleic Acid Isolation Kit	4463365
MagMAX™ FFPE DNA/RNA Ultra Kit	A31881
PureLink™ Genomic DNA Mini Kit	K182000
Nucleic acid quantification	
TaqMan® RNase P Detection Reagents Kit *(Recommended for DNA only)*	4316831
Qubit™ Fluorometer[1] and the Qubit™ dsDNA HS Assay Kit (DNA)	Q33226

[1] Qubit™ 2.0 Fluorometer and later are supported.

Bisulfite methylation workflow



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Before you begin

- We recommend adding unmethylated Lambda DNA to your DNA sample. The unmethylated Lambda DNA is used to estimate the bisulfite conversion rate.
- In addition, we also recommend using control DNA samples with known methylation rates to test alongside your sample of interest.
- The Ion AmpliSeq™ Methylation Panel for Cancer Research is a single pool (40 amplicons) and is designed for 125–175 bp amplicon length. Consequently, most cfDNA samples will not be appropriate for this panel.

Quantify input DNA

Use the procedure from the *Qubit™ dsDNA HS Assay Kits User Guide* (Pub. No. MAN0002326) to quantify the input DNA, including the control DNA and Lambda DNA.

Prepare bisulfite reagents

1. Prepare the CT Conversion reagent
   a. Mix the following reagents.

Reagent	Amount
Nuclease-free water	900 µL
Resuspension buffer	50 µL
Dilution Buffer	300 µL
Total	**1250 µL**

   b. Mix by vortexing or shaking for 10 minutes.
      - Trace amounts of undissolved material can remain.
      - Keep protected from light at room temperature until use.
      - Prepared reagent can be stored up to 1 week at –20°C.

2. Add 24 mL of 100% ethanol directly to the vial of Wash Buffer.
   Store at room temperature.

Bisulfite conversion

IMPORTANT!

- Do not elute in the tubes that are provided in the kit. Elute the final bisulfite converted DNA into 1.5 mL Eppendorf LoBind™ tubes.
- Use the standard cycling protocol.
- Do not use the Alternative cycling programs for longer DNA templates in the Appendix.
- We recommend using the MethylCode™ Bisulfite Conversion Kit. If other Bisulfite conversion kits are used, the yields and quality of the converted DNA can be too low for library preparation. If you are using another Bisulfite conversion kit, significant optimization can be required.
- Control DNA samples should be included in your experiment to compare library yields before sequencing, especially if FFPE samples are used.
- If FFPE samples are used, appropriate panel design amplicon lengths need to be utilized.
- Depending on the quality or quantity of the starting FFPE samples some FFPE samples can be too degraded to produce a successful library.

1. If needed, add sterile distilled water to the DNA sample to bring the volume up to 20 µl (10–100 ng of quantified DNA).
   - In general, for high-quality DNA, adjusting the quantity of input DNA to 10 ng before entering into library preparation is not necessary. If the quantity of input DNA is 10 ng, then the entire 10 µL bisulfite conversion elution volume should be used for library preparation.
   - If 100 ng of input DNA was used for the bisulfite conversion reaction, use 1 µL of the 10 µL bisulfite elution volume for library preparation and use the remaining volume for determining quantification and conversion efficiency. Alternatively, you can use all 10 µL and adjust the cycle number using the table in this document (see "Amplify the targets" on page 14).
   - Depending on the quality of the sample DNA, starting with 10 ng into bisulfite conversion can be sufficient to use all the eluted volume for your AmpliSeq reaction. If the quality of DNA is poor use additional DNA, if available, to enter into the bisulfite reaction.
   - For FFPE samples, it is recommended to start with ≥ 20 ng FFPE DNA for bisulfite conversion. We recommend starting with as much FFPE DNA into bisulfite conversion as possible up to 100 ng.

2. Add 0.33% Unmethylated Lambda DNA to your sample DNA before starting the bisulfite conversion reaction.
   - The bisulfite panel contains Lambda primers, which are used to determine bisulfite conversion rates based on the sequencing data. Unmethylated Lambda DNA must be spiked-in to your sample and control DNA before bisulfite conversion to determine conversion rates.
   - Lambda DNA is considered 1.52% the size of the human genome. For example, if you are adding 10 ng of your sample DNA, add 0.0005016 ng Lambda per 10 ng sample human DNA into the bisulfite reaction. [(10 ng * 0.0033) * 0.0152) = 0.0005016 ng] Measure your stock Lambda DNA with the Qubit™ dsDNA HS Assay Kit before diluting.

3. As an additional control, in a separate reaction, add 0.33% Promega Unmethylated Lambda DNA (D1521) to each control.

   Methylated Human gDNA control is expected to have an average of approximately ≥ 98% methylated CpG across all CpG sites.

4. In a PCR tube, add 130 µl of CT Conversion Reagent to the 20-µl DNA sample. Mix by flicking the tube or pipetting up and down.

5. Load the plate in the thermal cycler, then run the following program.

Stage	Temperature	Time
Denature DNA	98°C	10 minutes
Bisulfite conversion	64°C	2.5 hours
Hold	4°C	Up to 20 hours

6. Place a Spin Column in a Collection Tube, then add 600 µL of Binding Buffer to the column.

7. Add the sample from Step 5 to the Binding Buffer in the column. Close the cap, then mix by inverting several times.

8. Centrifuge at full speed (≥10,000 x g) for 30 seconds. Discard the flow-through.

9. Add 100 µL of Wash Buffer (prepared with ethanol as above) to the column, then centrifuge at full speed for 30 seconds. Discard the flow-through.

10. Add 200 µl of Desulphonation Buffer to the column, then let the column stand at room temperature for 15–20 minutes.

11. Centrifuge at full speed for 30 seconds. Discard the flow-through.

12. Add 200 µL of Wash Buffer that is prepared with ethanol to the column, then centrifuge at full speed for 30 seconds. Discard the flow-through.

13. Repeat the wash in Step 12 one more time, then transfer the spin column to a new, clean 1.5-mL Eppendorf LoBind™ tube tube.

14. Add 10 µL of Elution Buffer directly to the column matrix, incubate at room temperature for 1 minute, then centrifuge at full speed for 30 seconds to elute the DNA.

    The DNA is ready for immediate analysis, or can be stored at or below -20°C.

15. *(Optional)* If the starting amount of DNA was ≥ 50 ng, to roughly quantify the DNA, use the Qubit™ ssDNA Assay Kit (Cat. No. Q10212).

    General conversion efficiency is determined via the Lambda control during sequencing analysis.

Set up DNA target amplification reactions

Set up DNA target amplification reactions using one of the following procedures, depending on the number of primer pools in your Ion AmpliSeq™ panel.

Prepare DNA target amplification reactions—Ion AmpliSeq™ Methylation Panel for Cancer Research

Target amplification reactions can be assembled directly in a 96-well plate.

IMPORTANT! Primer pools and the 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly. We recommend PCR setup on ice or a cold block.

1. Prepare the Amplification Reaction Mix: combine the following components for the number of reactions that are required plus 20% overage, then pipet to mix thoroughly. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample DNA for multiple reactions.

Component	Volume
5X Ion AmpliSeq™ HiFi Mix (red cap)	• Input DNA >20 ng: 4 µL • Input DNA 10–20 ng: 3 µL • Input DNA <10 ng: 2–3 µL
5X Ion AmpliSeq™ Methylation Panel for Cancer Research Primer Pool	4 µL
Input DNA into Bisulfite conversion reaction (10–100 ng)	≤12 µL
Nuclease-free Water	to 20 µL

2. *(Optional)* If the mean read length is observed to be shorter than expected or shorter than the control, then reduce the amount of 5X Ion AmpliSeq™ HiFi Mix as described in the preceding table.

   Note: For low input or degraded samples, reducing the volume of the 5X Ion AmpliSeq™ HiFi Mix in the reaction results in increased mean read length during sequencing.

3. Seal the plate with a MicroAmp™ Clear Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

   Note: To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

4. Load the plate into a thermal cycler.

Proceed to "Amplify the targets" on page 14.

Prepare DNA target amplification reactions—single primer pool

For panels with 1 primer pool, target amplification reactions can be assembled directly in a 96-well plate.

IMPORTANT! Primer pools and the 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly. We recommend PCR setup on ice or a cold block.

1. Prepare the Amplification Reaction Mix: combine the following components for the number of reactions that are required plus 20% overage, then pipet to mix thoroughly. Add the following components to a single well of a 96-well PCR plate. Prepare a master mix without sample DNA for multiple reactions.

Component	Volume	
	2X primer pool	5X primer pool
5X Ion AmpliSeq™ HiFi Mix (red cap)	• Input DNA >20 ng: 4 µL • Input DNA 10–20 ng: 3 µL • Input DNA <10 ng: 2–3 µL	• Input DNA >20 ng: 4 µL • Input DNA 10–20 ng: 3 µL • Input DNA <10 ng: 2–3 µL
2X Ion AmpliSeq™ Primer Pool	10 µL	
5X Ion AmpliSeq™ Primer Pool		4 µL
Input DNA into Bisulfite conversion reaction (10–100 ng)	≤6 µL	≤12 µL
Nuclease-free Water	to 20 µL	to 20 µL

2. *(Optional)* If the mean read length is observed to be shorter than expected or shorter than the control, then reduce the amount of 5X Ion AmpliSeq™ HiFi Mix as described in the preceding table.

   Note: For low input or degraded samples, reducing the volume of the 5X Ion AmpliSeq™ HiFi Mix in the reaction results in increased mean read length during sequencing.

3. Seal the plate with a MicroAmp™ Clear Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

   Note: To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

4. Load the plate into a thermal cycler.

Proceed to "Amplify the targets" on page 14.

Prepare DNA target amplification reactions—2 primer pools

If you are using a DNA panel with 2 primer pools, set up two 10-µL amplification reactions, then combine them after target amplification to give a volume of 20 µL.

IMPORTANT! Primer pools and the 5X Ion AmpliSeq™ HiFi Mix are viscous. Pipet slowly and mix thoroughly. We recommend PCR setup on ice or a cold block.

1. Prepare the Amplification Reaction Mix: combine the following components for the number of reactions that are required plus 20% overage, then pipet to mix thoroughly. Add the following components to a 1.5-mL Eppendorf LoBind™ tube. Prepare a master mix without sample DNA for multiple reactions.

Component	Volume	
	2X primer pool	**5X primer pool**
5X Ion AmpliSeq™ HiFi Mix (red cap)	• Input DNA >20 ng: 5 µL • Input DNA 10–20 ng: 3.75 µL • Input DNA <10 ng: 2.5–3.75 µL	• Input DNA >20 ng: 4.5 µL • Input DNA 10–20 ng: 3.375 µL • Input DNA <10 ng: 2.25–3.375 µL
Input DNA into Bisulfite conversion reaction (10–100 ng)	⩽7.5 µL	⩽13.5 µL
Nuclease-free Water	to 12.5 µL	to 18 µL

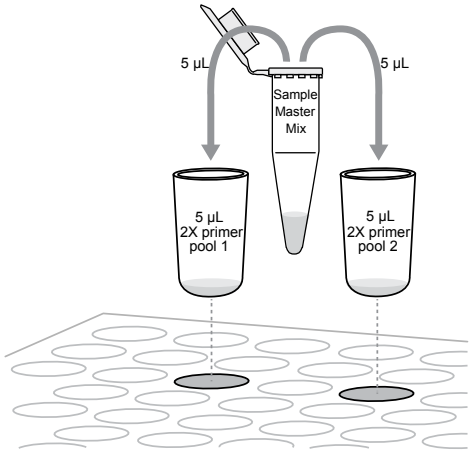
2. *(Optional)* If the mean read length is observed to be shorter than expected or shorter than the control, then reduce the amount of 5X Ion AmpliSeq™ HiFi Mix as described in the preceding table.

   Note: For low input or degraded samples, reducing the volume of the 5X Ion AmpliSeq™ HiFi Mix in the reaction results in increased mean read length during sequencing.

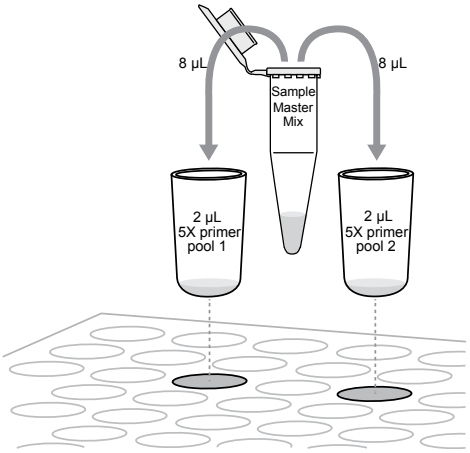
3. Mix thoroughly by pipetting up and down 5 times, then transfer sample-specific master mixes to 2 wells of a 96-well PCR plate:

   - For 2X primer pools, transfer 5 µL of master mix into 2 wells. Add 5 µL of primer pool 1 into the first well, and 5 µL of primer pool 2 to the second well.
   - For 5X primer pools, transfer 8 µL of master mix into 2 wells. Add 2 µL of primer pool 1 into the first well, and 2 µL of primer pool 2 to the second well.

2-pool panels at 2X concentration:



2-pool panels at 5X concentration:



4. Seal the plate with a MicroAmp™ Clear Adhesive Film, then place a MicroAmp™ Compression Pad on the plate.

   Note: To prevent evaporation during target amplification, use an applicator tool to press the film securely around each reaction well and around the perimeter of the plate.

5. Load the plate into a thermal cycler.

Proceed to "Amplify the targets" on page 14.

Amplify the targets

1. Determine the number of amplicons for your panel.

   Note: The number of amplicons will **not** be the total number of primers divided in half.

2. Run the following program.

Stage	Step	Temperature	Time
Hold	Activate the enzyme	99°C	2 minutes
Cycle; set number according to the following tables	Denature	99°C	15 seconds
	Anneal and extend	60°C	4 minutes
Hold	—	10°C	Hold

Total amplicons per pool	Number of amplification cycles	
	High quality DNA	Low quality DNA (FFPE DNA or cfDNA)
Ion AmpliSeq™ Methylation Panel for Cancer Research only[1]		
40	28	31
All other bisulfite panels		
12–24	29	32
25–48	28	31
49–96	27	30
97–192	26	29
193–384	25	28
385–768	24	27

[1] The Ion AmpliSeq™ Methylation Panel for Cancer Research pool has 40 amplicons (single pool 5X) and is designed for 125-175 bp amplicon length, consequently, most cfDNA samples will not be suitable for this panel.

Cycle number recommendations in the preceding table are based on 10 ng of DNA input. Adjust cycle number from the preceding table for lower or higher DNA input:

Amount of DNA starting material	Adjustment to cycle number
1 ng (300 copies)	+3
10 ng (3,000 copies)	0
100 ng (30,000 copies)	–3

STOPPING POINT Target amplification reactions can be stored at 10°C overnight on the thermal cycler. For longer term, store at –20°C.

Combine target amplification reactions (2-pool DNA panels only)

1. Centrifuge the plate briefly to collect contents, then carefully remove the plate seal.
2. For each sample, combine the 10-µL target amplification reactions. The total volume for each sample should be ~20 µL.

Partially digest amplicons

IMPORTANT! FuPa Reagent is viscous. Pipet slowly and mix thoroughly. Perform this step on ice or a cold block, then quickly proceed to incubation.

1. One-primer pool panel: centrifuge briefly to collect the contents, then carefully remove the plate seal.
2. **Add 2 µL of FuPa Reagent** (brown cap) to each amplified sample. The total volume is ~22 µL.
3. Seal the plate with a clear adhesive film, vortex thoroughly, then centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
4. Place a compression pad on the plate, load in the thermal cycler, then run the following program:

Temperature	Time
50°C	10 minutes[1]
55°C	10 minutes[1]
60°C	20 minutes
10°C	Hold (for up to 1 hour)

[1] Increase to 20 minutes for panels over 1,536 amplicons

STOPPING POINT Store plate at –20°C for longer periods.

Ligate adapters to the amplicons and purify

When sequencing multiple libraries on a single chip, you *must* ligate a different barcode adapter to each library.

IonCode™ adapters are provided at the appropriate concentration and include forward and reverse adapters in a single well. No further handling is necessary.

Ion Xpress™ adapters require handling and dilution as described in the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

IMPORTANT! When handling barcoded adapters, be careful to avoid cross-contamination by changing gloves frequently and opening one tube at a time.

Perform the ligation reaction

1. If there is visible precipitate in the Switch Solution or the tube cap after thawing, vortex or pipet up and down at room temperature to resuspend before pipetting.
2. Briefly centrifuge the plate to collect the contents.
3. Carefully remove the plate seal, then add the following components in the order that is listed to each well containing digested amplicons. If preparing multiple non-barcoded libraries, a master mix of Switch Solution and adapters can be combined before addition.

   IMPORTANT! Add the DNA Ligase last. Do not combine DNA Ligase and adapters before adding to digested amplicons.

Order of addition	Component	Volume
1	Switch Solution (yellow cap)	4 µL
2	Adapters (Ion Torrent™ Dual Barcode Adapters, IonCode™ Adapters, *or* diluted Ion Xpress™ barcode adapter mix (for barcoded libraries))	2 µL
3	DNA Ligase (blue cap)	2 µL
—	**Total volume** (including ~22 µL of digested amplicon)	**~30 µL**

4. Seal the plate with a new MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.

5. Place a MicroAmp™ Compression Pad on the plate, load in the thermal cycler, then run the following program:

Temperature	Time
22°C	30 minutes
68°C	5 minutes
72°C	5 minutes
10°C	Hold (for up to 24 hours)

STOPPING POINT Samples can be stored for up to 24 hours at 10°C on the thermal cycler. For longer periods, store at –20°C.

Purify the library

IMPORTANT! Bring Agencourt™ AMPure™ XP Reagent to room temperature and vortex thoroughly to disperse the beads before use. Pipet the solution slowly.

Do NOT substitute a Dynabeads™-based purification reagent for the Agencourt™ AMPure™ XP Reagent.

1. Briefly centrifuge the plate to collect the contents in the bottom of the wells.
2. Carefully remove the plate seal, then add 30 µL (1X sample volume) of Agencourt™ AMPure™ XP Reagent to each library. Pipet up and down 5 times to mix the bead suspension with the DNA thoroughly.

   Note: Visually inspect each well to ensure that the mixture is homogeneous.
3. Incubate the mixture for 5 minutes at room temperature.
4. Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet, then incubate for 5 minutes or until the solution clears. Carefully remove, then discard the supernatant without disturbing the pellet.
5. Add 150 µL of freshly prepared 70% ethanol, then move the plate side-to-side in the two positions of the magnet to wash the beads. Carefully remove, then discard the supernatant without disturbing the pellet.
6. Repeat step 5 for a second wash.
7. Ensure that all ethanol droplets are removed from the wells. Keeping the plate in the magnet, air-dry the beads at room temperature for 5 minutes. Do not overdry.

   IMPORTANT! Residual ethanol drops inhibit library amplification. If needed, centrifuge the plate and remove remaining ethanol before air-drying the beads. Under conditions of low relative humidity the beads air-dry rapidly. Do not overdry.
8. Add 50 µL of low TE.
9. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.

10. Incubate at room temperature for 2 minutes.
11. Place the plate on the magnet for at least 2 minutes.
12. Transfer 50 µL volume to a new well on the plate (do not disturb the pellet).
13. Add 50 µL (1X sample volume) of Agencourt™ AMPure™ XP Reagent.
14. Pipet up and down 5 times to thoroughly mix the bead suspension with the DNA.
15. Repeat steps 3–7.

Proceed to "Elute the library" on page 18, immediately. Not a stopping point.

IMPORTANT! Do not overdry the beads.

Quantify the unamplified library by qPCR

Elute the unamplified Ion AmpliSeq™ library, then determine the concentration by qPCR with the Ion Library TaqMan® Quantitation Kit (Cat. No. 4468802). Unamplified libraries typically have yields of 100–500 pM. After quantification, determine the dilution factor that results in a concentration of ~100 pM, which is appropriate for template preparation using an Ion template kit.

Elute the library

1. Remove the plate with purified libraries from the plate magnet, then **add 50 µL of Low TE** to the pellet to disperse the beads.
2. Seal the plate with MicroAmp™ Clear Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets. Alternatively, mix by pipetting at least half the total volume up and down at least 5 times before sealing the plate.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate on the magnet for at least 2 minutes.
5. Prepare a 100-fold dilution for quantification. Remove 2 µL of supernatant, containing the library, then combine with 198 µL of Nuclease-free Water.

Quantify library by qPCR and calculate the dilution factor

Determine the concentration of each Ion AmpliSeq™ library by qPCR with the Ion Library TaqMan® Quantitation Kit using the following steps. Analyze each sample, standard, and negative control in duplicate 20-µL reactions.

1. Prepare three 10-fold serial dilutions of the *E. coli* DH10B Control Library (~68 pM; from the Ion Library TaqMan® Quantitation Kit) at 6.8 pM, 0.68 pM, and 0.068 pM. Mark these tubes as standards, then use these concentrations in the qPCR instrument software.

2. Prepare reaction mixtures. For each sample, control, and standard, combine 20 µL of 2X Ion Library qPCR Master Mix and 2 µL of Ion Library TaqMan® Quantitation Assay, 20X , then mix thoroughly. Dispense 11-µL aliquots into the wells of a PCR plate.

3. Add 9 µL of the diluted (1:100) Ion AmpliSeq™ library or 9 µL of each control dilution to each well (two wells per sample as noted before), for a total reaction volume of 20 µL.

4. Program your real-time instrument as follows:
   a. Enter the concentrations of the control library standards.
   b. Select ROX™ Reference Dye as the passive reference dye.
   c. Select a reaction volume of 20 µL.
   d. Select FAM™ dye/MGB as the TaqMan® probe reporter/quencher.
   e. The Ion Library qPCR Master Mix can be used on various instruments, as listed in the following table. The fast cycling program was developed using the StepOnePlus™ System in Fast mode.

IMPORTANT! When quantifying libraries made from panels with 275-bp or 375-bp designs, use standard qPCR cycling. Fast cycling can result in inaccurate quantification.

Real-time PCR System	Reaction plate	Run mode	Stage	Temperature	Time
7500 Fast	96-well Fast	Fast	Hold (UDG incubation)	50°C	2 min
7900 HT 7900 HT Fast	96-well Fast 384-well Standard		Hold (polymerase activation)	95°C	20 sec
ViiA™ 7			Cycle (40 cycles)	95°C	1 sec
QuantStudio™ 3, 5, or 7					
StepOne™ StepOnePlus™	48-/96-well Fast			60°C	20 sec
7300	96-well Standard	Standard	Hold (UDG incubation)	50°C	2 min
7500			Hold (polymerase activation)	95°C	2 min

Real-time PCR System	Reaction plate	Run mode	Stage	Temperature	Time
7900 HT 7900 HT Fast	96-well Standard	Standard	Cycle (40 cycles)	95°C	15 sec
ViiA™ 7				60°C	1 min
QuantStudio™ 3, 5, or 7					

5. Following qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ library by multiplying the concentration that is determined with qPCR by 100.

6. Based on the calculated library concentration, determine the dilution that results in a concentration of ~50–100 pM.

   For example:

   - The undiluted library concentration is 300 pM.
   - The dilution factor is 300 pM/100 pM = 3.
   - Therefore, 10 µL of library mixed with 20 µL of Low TE (1:3 dilution) yields approximately 100 pM.

7. Dilute library to ~50–100 pM, combine, then proceed to template preparation, or store libraries as described below.

(Optional) Combine amplicon libraries

Multiple strategies for combining libraries are available. See Appendix C, "Strategies for combining Ion AmpliSeq™ libraries", in the *Ion AmpliSeq™ Library Kit Plus User Guide* (Pub. No. MAN0017003).

Store libraries

You can store libraries at 4–8°C for up to 1 month. For longer lengths of time, store at –30°C to –10°C.

About Planned Runs

IMPORTANT! For more information on creating a Planned Run in Torrent Suite™ Software, including a complete description of each field in the **Create Plan** workflow bar, see the *Torrent Suite™ Software Help*, available by clicking the **Help** button in the software.

hg19_Lambda reference sequence

Ensure that the hg19_Lambda reference sequence has been uploaded to Torrent Suite™ Software. For additional information, see the *Torrent Suite™ Software 5.12 Help*.

Note: The hg19_Lambda reference sequence is not the same as the hg19 reference sequence. The hg19_Lambda reference sequence includes the Lambda reference sequence in addition to the standard hg19 reference sequence. For additional information, see the *Torrent Suite™ Software 5.12 Help*.

Create a custom Planned Run template

We recommend setting up a customized Planned Run template for reuse when the same conditions will be used for multiple runs. For more information about creating Planned Runs manually or from the generic application template, see the online help available within the Torrent Suite™ Software.

1. Sign in to the Torrent Browser for the Torrent Server connected to your Ion Chef™ System.
2. Under the **Plan** tab, in the **Templates** screen, in the favorites pane, select **AmpliSeq DNA**, then click **Add New Template**.
3. Fill in the key fields using the values in the following table.

Planned Run wizard key fields

Field name	Parameter
Ion Reporter step	
Ion Reporter Account	None
Existing Workflow	N/A
Sample Grouping	Self
Ion Reporter Upload Options	N/A
Research Application step	
Research Application	DNA
Target Technique	AmpliSeq DNA
Kits step	
Instrument	Ion GeneStudio™ S5 System
Chip Type	Ion 520™ Chip or Ion 530™ Chip
Sample Preparation Kit	Optional
Control Sequence	Leave blank

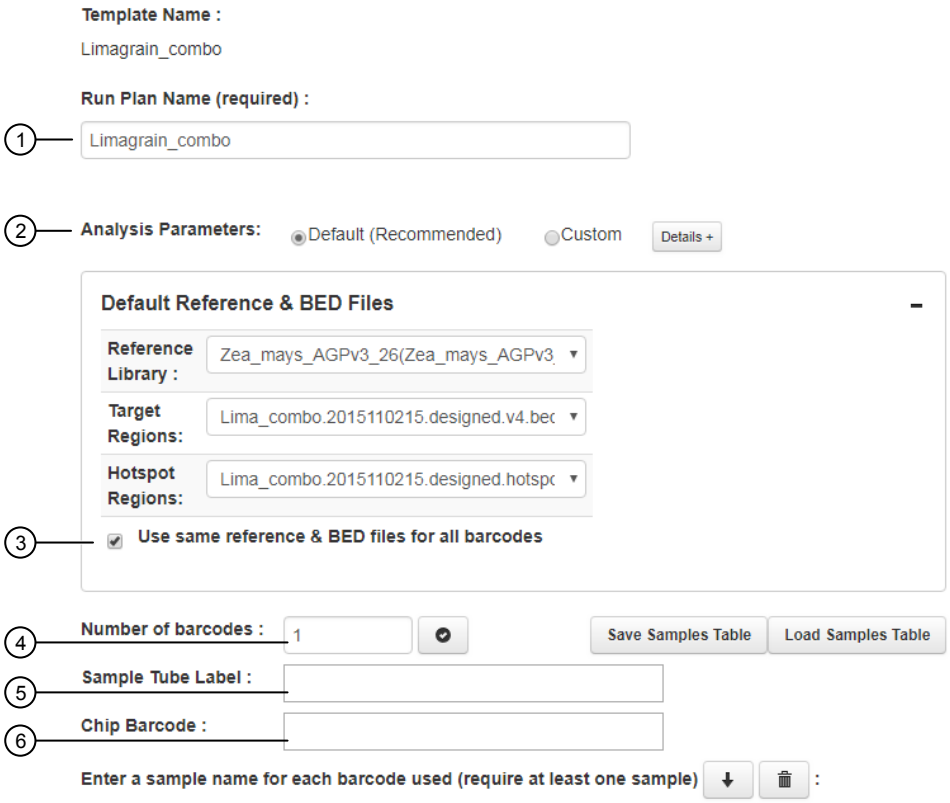
Field name	Parameter
Library Kit Type	Leave blank
Barcode Set	Select the barcode set that was used for library preparation.
Template Kit	1. Select **IonChef**. 2. Select **Ion 520™ & Ion 530™ ExT Kit – Chef**.
Flows	500
Sequencing Kit	Select **Ion S5 ExT Sequencing Kit**.
Mark as Duplicate Reads	Leave deselected
Enable Realignment	Leave deselected
Advanced Settings	Use Recommended Defaults
Plugins step	
Plugins	Select **methylation_analysis**, click **Configure**, then ensure that the parameters are correct.
Projects step	
Projects	Select or add a project within which to group your run data. You can include runs in multiple projects, and remove runs from a project at any time.
Plan step	
Run Plan Name	Enter a name for the run.
Analysis parameters	Default
Reference Library	hg19_Lambda
Target regions	Contact your sales representative.
Hotspot regions	N/A
Number of barcodes	Enter the number of barcodes.
Sample Tube Label (Required)	Enter or scan the barcode of each Ion Chef™ Library Sample Tube that you use to load a sample into the Ion Chef™ Instrument. **IMPORTANT!** You must scan or enter the barcode of each Ion Chef™ Library Sample Tube used on the Ion Chef™ Instrument.
Chip Barcode	Enter or scan the chip barcode.
Enter a sample name for each barcode used	Is Ion Reporter™ Uploader enabled? **No** – Scan the barcode of the Ion Chef™ Library Sample Tube into the "Sample Name" and "Sample Tube Label" fields for the specific sample.

Field name	Parameter
Monitoring Thresholds	Set thresholds for Bead Loading, Usable Sequence, and Key Signal. In the Torrent Browser **Monitor ▸ Runs in Progress** tab, an alert is displayed if the values for a run fall below the selected thresholds.
Add a note	Enter notes for the planned run.
Add LIMS Meta Data	Enter meta data for the planned run.

Create a Planned Run from a template

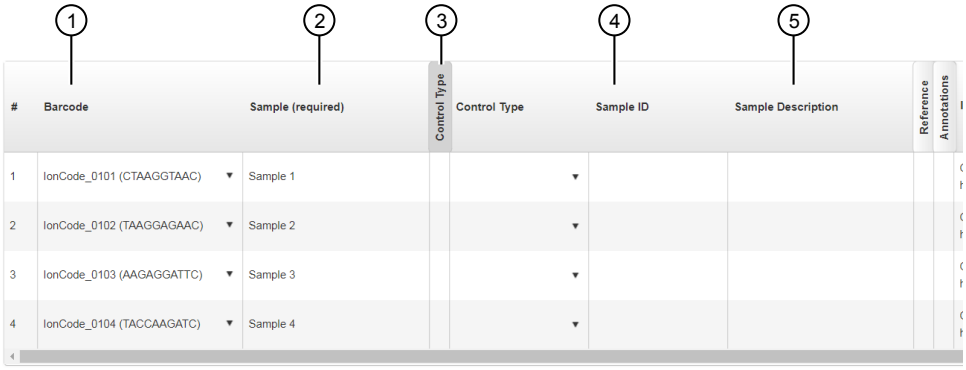
1. Sign in to the Torrent Browser for the Torrent Server that is connected to your Ion Chef™ System.
2. Click **Plan tab ▸ Templates**.
3. In the list, click your Planned Run template. Alternatively, in the row of the template click ⚙ ▾ then select **Plan Run**.
   The create plan wizard opens to the **Plan** tab.

4. Enter or select the following information. Row numbers in the table correspond to the call-outs in the following figure.



Callout	Field	Action
1	Run Plan Name	Enter a Run Plan name.
2	Analysis Parameters	Ensure the Default (Recommended) radio button is selected.
3	Use same reference & BED files for all barcodes	Ensure that the checkbox is selected.
4	Number of barcodes	Enter the number of barcodes that will be used in this run, then click the ✔ button to the right of this field.
5	Sample Tube Label	Enter or scan the barcode of the Ion Chef™ sample tube that will be used in the run.
6	Chip Barcode	No entry required.

5. Enter sample information. Row numbers in the table correspond to the callouts in the following figure.



Callout	Field[1]	Action
1	Barcode	For each sample select the **Barcode** that will identify it from the dropdown list.
2	Sample Name	Accept the auto-populated sample names or click in a field, then enter a unique sample name. We recommend sample names (either auto-populated or user defined) be unique even between runs. **IMPORTANT!** Do NOT include spaces in sample names.
3	Control Type (expanded)	Select No Template Control from the dropdown list to designate a sample as a no template control.
4	Sample ID	*(Optional)* Click in the field, then enter a sample ID.
5	Sample Description	*(Optional)* Click in the field, then enter a sample description.

[1] Click vertical column headers (Control Type, Reference, Annotations) to reveal additional columns.

6. Click **Plan Run**.

Guidelines for templating and sequencing

Proceed to template preparation and sequencing using the following kits.

Template System	Sequencer	Kit	User Guide
Ion Chef™	Ion GeneStudio™ S5 Sequencers	Ion 520™ & Ion 530™ ExT Kit – Chef (Cat. No. A30670)	*Ion 520™ & Ion 530™ ExT Kit – Chef User Guide* (Pub. No. MAN0015805)

IMPORTANT! For the sequencing run, set the number of flows to 500.

Note:

- Adjust the concentration of your combined library to 10–40 pM.
- Use 50 µL of your pooled library (10–40 pM).
- You can only run one chip per templating run when using the standard ExT protocol.

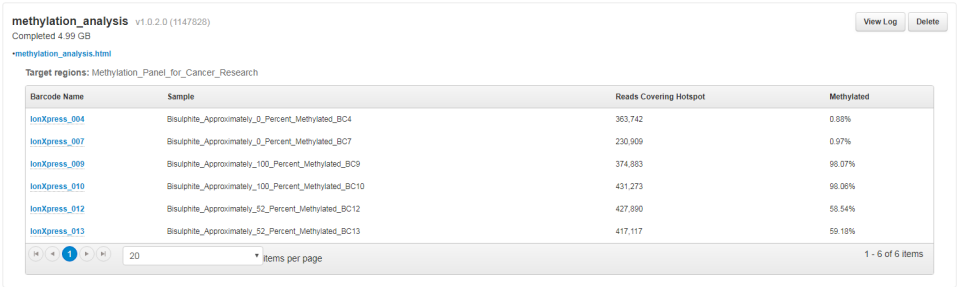
Review sequencing run results

The methylation_analysis plugin performs alignment and methylation calling for amplicons on both the Watson (W) and Crick (C) strands. Each amplicon can have zero, one or more designated CpG targets (hotspots) of interest. A summary report shows each barcode name along with the sample name, the total number of reads covering the target CpGs, and the percentage of those reads that are methylated. In addition, for each barcode, text files are generated giving the number of methylated reads, unmethylated reads, and percent methylation for each amplicon. There are separate text files for the designated target CpGs, all CpGs in the amplicon insert and all non-CpG Cs in the amplicon insert.

1. After the sequencing run is complete, in the **Data** tab, click **Completed Runs & Reports**.

2. In the list of runs, find the run of interest, then click the link in the **Report Name** column.
   - Only the unaligned reads section is used for analysis. Disregard the aligned reads section.
   - The methylation_analysis plugin incorporates a modified version of the publicly available bisulfite alignment program Bismark to perform bisulfite aware alignment.
   - If the plugin was selected during run planning, no configuration is required.
   - The first time the plugin is run for a particular reference, it must set up bisulfite converted versions of the reference for alignment. This can take several extra hours.

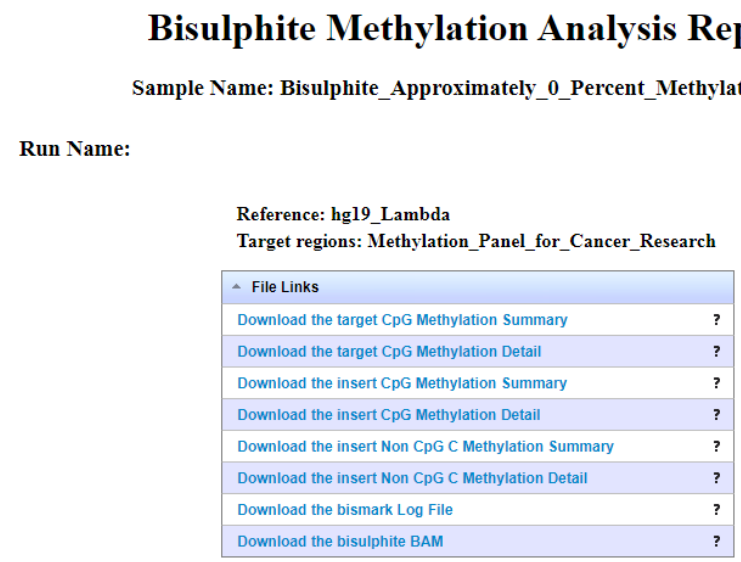
3. In the left navigation menu, click **methylation_analysis** to view the plugin summary.



4. *(Optional)* Click **methylation_analysis.html**, then click any of the following to download the results for that file.
   - **Watson Coverage Analysis Results**: coverageAnalysis results for the Watson strand.
   - **Crick Coverage Analysis Results**: coverageAnalysis results for the Crick strand.
   - **Download a ZIP report summary**: Downloads a ZIP file of the results.

   Note: The Watson and Crick coverageAnalysis results pages look identical to standard coverageAnalysis, but use the bisulfite aligned BAM files.

5. Click a specific barcode to show the analysis report for that barcode, then click a report title to download individual methylation calling results files, the Bismark log file, and the bisulfite aligned BAM file.



File	Description
Target CpG methylation summary	Gives the average number of methylated and unmethylated reads across all target CpGs contained in each amplicon insert along with the average percent methylation. The target CpGs are specified in the BED file for each amplicon. This file has no column for position as the values are averages.
Target CpG methylation detail	Gives the number of methylated and unmethylated reads at each target CpG contained in each amplicon insert along with the average percent methylation at each target CpG. The target CpGs are specified in the BED file for each amplicon.
Insert CpG methylation summary	Gives the average number of methylated and unmethylated reads across all CpGs contained in each amplicon insert along with the average percent methylation. This file has no column for position as the values are averages.

File	Description
Insert CpG methylation detail	Gives the number of methylated and unmethylated reads at each CpG contained in each amplicon insert along with the average percent methylation at each CpG.
Insert non-CpG C methylation summary.	Gives the average number of methylated and unmethylated reads across all non-CpGs Cs contained in each amplicon insert along with the average percent methylation. This file has no column for position as the values are averages.
Insert non-CpG C methylation detail	Gives the number of methylated and unmethylated reads at each non-CpG C contained in each amplicon insert along with the average percent methylation at each non-CpG C.
Bismark Log File	Contains events and statistics from the bisulfite alignment performed by Bismark.
Bisulfite BAM	The bisulfite aligned BAM file .

Calculate bisulfite conversion rate

The bisulfite conversion rate measures the efficiency of the bisulfite conversion reaction.

The methylation_analysis plugin screens the sequencing data to determine the number of methylated residues in the unmethylated Lambda DNA (Unmethylated Lambda DNA Promega D1521), which was added to your sample or control gDNA before bisulfite conversion. Two sets of primers corresponding to Lambda DNA are included in the bisulfite panel, which allows the Lambda DNA to be sequenced with your samples and control DNA. After bisulfite conversion is performed, theoretically every C residue in the unmethylated Lambda control DNA should be converted to a T. The methylation_analysis plugin counts the number of C residues that are present in the sequence to determine the percent of the sequence that is methylated, as after the bisulfite conversion reaction, the only C residues that are present are methylated in the original sample. This value is shown in the percent.ME column for each sample.

1. Open the **Insert CpG Methylation Summary** file.

2. Calculate the bisulfite conversion rate as follows.
    a. Calculate the average of the Watson (L01 W) and Crick (L01 C) percent.ME values (percent methylation) for the unmethylated Lambda DNA in each sample.

    b. Subtract the average from 100 to obtain the bisulfite conversion rate for each sample and control.

    For example, if the percent.ME values for the Lambda Watson (L01 W) and Lambda Crick (L01 C) strands are 0.65 and 0.34, respectively, the average is 0.495. Subtracting 0.495 from 100 gives a bisulfite conversion efficiency of 99.505.

    The bisulfite conversion rate should be >99%.

Calculate total methylation reads per amplicon

For each amplicon listed (amp.id), add the numbers under the columns for methylated reads (reads.ME) and unmethylated reads (reads.UM) together to obtain the total methylated reads per amplicon.

For example, if the reads.ME value for the amplicon is 707 and the reads.UM value for the same amplicon is 27 the sum would be 734, which is the total methylation reads for this amplicon. The total methylation reads per amplicon should be ≥50.

Criteria for a successful run

- A successful run requires ≥ 50 total methylation reads per individual amplicon.
- Chip loading in the range of ~43-57% has been observed in successful 530 chip runs. Low chip loading is expected for this application.
- Ion AmpliSeq™ Methylation Panel for Cancer Research has an example data set posted with further metrics listed for guidance (**AmpliSeq.com**).

Life Technologies Corporation | 5781 Van Allen Way | Carlsbad, CA 92008
For descriptions of symbols on product labels or product documents, go to **thermofisher.com/symbols-definition**.

Revision history: Pub. No. MAN0017662

Revision	Date	Description
B.0	3 June 2019	Corrected Sequencing kit parameter in "Planned Run wizard key fields" on page 21.
A.0	29 January 2019	New document

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3 June 2019