

Bisulfite methylation library production and analysis using the Ion AmpliSeq™ Kit for Chef DL8

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The bisulfite method is the most commonly used technique for identifying specific methylation patterns within a DNA sample. This protocol combines a one day bisulfite kit protocol with the Ion AmpliSeq™ Kit for Chef DL8 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™ Kit for Chef DL8 and Ion AmpliSeq™ panels are used to prepare amplicon libraries of the bisulfite-treated DNA on the Ion Chef™ System, which automates the preparation of libraries. Libraries are sequenced using 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™ Kit for Chef DL8, see the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432).

Note: For safety and biohazard guidelines, see the “Safety” appendix in the following product documentation: *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Required kits

Ion AmpliSeq™ Kit for Chef DL8 (library preparation)

IMPORTANT! Store all consumables and cartridges under the recommended conditions and in an upright position. Ion AmpliSeq™ Chef Solutions DL8 cartridges are shipped at ambient temperature, but need to be stored at 2°C to 8°C upon arrival.

The Ion AmpliSeq™ Kit for Chef DL8 (Cat. No. A29024) contains materials required for performing 4 Ion Chef™ runs, with up to 8 Ion AmpliSeq™ libraries prepared per run. Upon arrival, inspect all consumables and contact Technical Support if any of the components have been damaged during shipping.

Component	Part No.	Quantity per kit	Storage
Ion AmpliSeq™ Chef Reagents DL8	A29025	4 cartridges	-30°C to -10°C
Ion AmpliSeq™ Chef Solutions DL8	A29026	4 cartridges	2°C to 8°C ^[1]
Ion AmpliSeq™ Chef Supplies DL8 (per insert) <ul style="list-style-type: none">• Ion AmpliSeq™ Tip Cartridge L8• PCR Frame Seal• PCR Plate Frame• Enrichment Cartridge	A29027	1 box with 4 inserts	15°C to 30°C
IonCode™ 0101–0132 in 96 Well PCR Plates (dried) Set includes 4 PCR plates: <ul style="list-style-type: none">• IonCode™ 0101–0108 in 96 Well PCR Plate (red)• IonCode™ 0109–0116 in 96 Well PCR Plate (yellow)• IonCode™ 0117–0124 in 96 Well PCR Plate (green)• IonCode™ 0125–0132 in 96 Well PCR Plate (blue)	A29028	1 set of 4 plates	15°C to 30°C

^[1] Ion AmpliSeq™ Chef Solutions DL8 cartridges are shipped at ambient temperature, but need to be stored at 2°C to 8°C upon arrival.

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

(continued)

Component	Amount
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](https://www.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](https://www.ampliseq.com), then order the amplification panel.

Required materials not supplied

Unless otherwise indicated, all materials are available through thermofisher.com. "MLS" indicates that the material is available from fisherscientific.com or another major laboratory supplier.

Item	Source
Ion Chef™ System	4884177
Agencourt™ AMPure™ XP Kit	NC9959336 , NC9933872
Unmethylated Lambda DNA	Promega D1521
CpG Methylated Jurkat Genomic DNA, or equivalent quality methylated or unmethylated human gDNA control	SD1121
P20 and P200 µL pipettors, and filtered tips	fisherscientific.com
<i>(Optional)</i> Non-interruptible Power Supply (UPS) ^[1]	MLS
Nuclease-free Water	AM9932
Ethanol, Absolute, Molecular Biology Grade	BP2818500 (fisherscientific.com)
Low TE (10 mM Tris, 0.1 mM EDTA, pH 8.0)	50-843-203 (fisherscientific.com)
Ion 520™ & Ion 530™ ExT Kit – Chef	A30670
Ion 520™ Chip Kit or Ion 530™ Chip Kit	A27762 A27764
MicroAmp™ Clear Adhesive Film	4306311
MicroAmp™ Optical Film Compression Pad	4312639
Thermal Cycler <i>or</i> equivalent	4375786
Choose one of the following sets, or equivalent.	
DynaMag™-96 Side Magnet	12331D
MicroAmp™ Optical 96-Well Reaction Plate	N8010560
MicroAmp™ Clear Adhesive Film	4306311
Eppendorf™ DNA LoBind™ Microcentrifuge Tubes, 1.5-mL	13-698-791 (fisherscientific.com)
DynaMag™-2 Magnet	12321D

^[1] For laboratories that experience frequent power outages or line voltage fluctuations, we recommend that you use a non-interruptible power supply that provides 2500 W output or higher.

Recommended materials and equipment

Unless otherwise indicated, all materials are available through [thermofisher.com](https://www.thermofisher.com).

Item	Source
Qubit™ ssDNA Assay Kit	Q10212
Additional equipment	
Fisher Scientific™ Mini Plate Spinner Centrifuge, or equivalent 96-well plate centrifuge	14-100-143 (fisherscientific.com)
Recommended for nucleic acid isolation	
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	AM1975
RecoverAll™ Multi-Sample RNA/DNA Workflow	A26069 and A26135
MagMAX™ FFPE DNA/RNA Ultra Kit	4463365
PureLink™ Genomic DNA Mini Kit	K182001
Recommended for nucleic acid quantification	
TaqMan™ RNase P Detection Reagents Kit (<i>Recommended for gDNA</i>)	4316831
Qubit™ Fluorometer ^[1] and the Qubit™ dsDNA HS Assay Kit (DNA)	Q33238 Q32851, Q32854
(<i>Optional</i>) Ion Library TaqMan™ Quantitation Kit ^[2]	4468802

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

^[2] Recommended for checking concentration of combined library.

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.
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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 step 8 of “Start the Ion Chef™ run” on page 12).
 - 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. $[(10 \text{ ng} * 0.0033) * 0.0152] = 0.0005016 \text{ ng}$ 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 ($\geq 10,000 \times g$) 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.
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 $\geq 50 \text{ ng}$, 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.

Prepare gDNA

You need to run 8 samples per run on the Ion Chef™ System.

Dilute the bisulfite converted DNA.

Component	Volume
Eluted bisulfite converted DNA (10-100 ng)	10 μL
Nuclease-free water	to 15 μL
Total	15 μL

Thaw the reagents and prepare the instrument

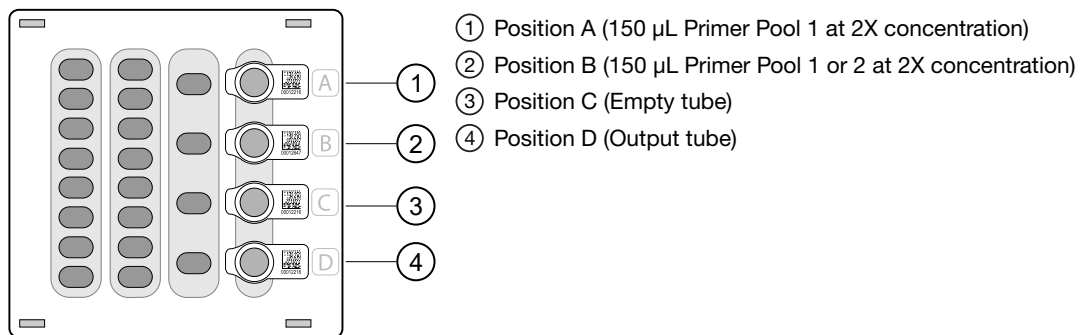
- Before the run, thaw one Ion AmpliSeq™ Chef Reagents DL8 cartridge at room temperature for 20 minutes.
- Thaw the Ion AmpliSeq™ primer pools.
- If not performed after a previous run, unload and clean the Ion Chef™ Instrument.

- Confirm that the Ion Chef™ Instrument has a connection to the Torrent Server. On the Ion Chef™ home touchscreen, touch **Settings ▶ Torrent Server** to view the connection status of your instrument.

Note: If the instrument is not connected, see the *Ion Chef™ and Torrent Server Network Setup User Guide* (Pub. No. MAN0013444) for instructions on how to configure a direct or indirect network connection of the Ion Chef™ Instrument to a Torrent Server.

Add Ion AmpliSeq™ 2X Primer Pools to Positions A and B of the Reagents cartridge

1. Uncap all 4 tubes in Positions A, B, C, and D in the Ion AmpliSeq™ Chef Reagents DL8 cartridge. Save the caps.



2. Add primer panels to the Reagents cartridge using the following guidelines:

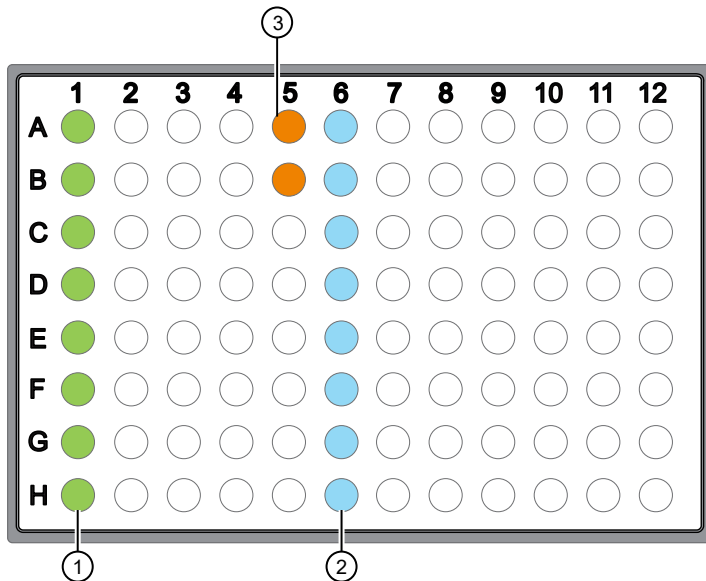
If you are using	Action
Chef-Ready panels	
1 or 2 primer pools	<ol style="list-style-type: none"> 1. Vortex the Primer Pool Tubes to mix, then centrifuge. 2. Remove the caps, then replace the tubes in Positions A and B of the Ion AmpliSeq™ Chef Reagents DL8 cartridge with the Chef-Ready panel tubes.
Ion AmpliSeq™ 2X panels – Aliquot only.	
1 primer pool	<ol style="list-style-type: none"> 1. Pipet 150 µL of the 2X Primer Pool into each of the Position A and Position B tubes.
2 primer pools	<ol style="list-style-type: none"> 1. Pipet 150 µL of the 2X Primer Pool 1 into the Position A tube. 2. Pipet 150 µL of the 2X Primer Pool 2 into the Position B tube.
Ion AmpliSeq™ 5X panels – Dilute and Aliquot.	
Note: The Ion AmpliSeq™ Methylation Panel for Cancer Research is a 5X panel with 1 primer pool.	
1 primer pool	<ol style="list-style-type: none"> 1. Dilute to 2X by adding 120 µL of the 5X Primer Pool to 180 µL of Nuclease-free Water. 2. Pipet 150 µL of the 2X Primer Pool into each of the Position A and B tubes.

(continued)

If you are using	Action
2 primer pools	<ol style="list-style-type: none"> Pipet 60 µL of the 5X Primer Pool 1 into the Position A tube. Pipet 60 µL of the 5X Primer Pool 2 into the Position B tube. Pipet 90 µL of Nuclease-free Water into each of the Position A and Position B tubes. Using a new tip for each tube, pipet up and down 5 times to mix.

Add DNA to the IonCode™ PCR plate

- Remove, then discard the plate seal from an IonCode™ 96-well PCR Plate.
- Remove the plate seal from an IonCode™ 96-well PCR Plate.
- Pipet 15 µL of each diluted bisulfite converted DNA sample (10–100 ng starting DNA), into wells A1 to H1 of the plate.



- Each column 1 well contains 15 µL of diluted bisulfite converted DNA sample (10–100 ng starting DNA), or Nuclease-free Water as non-template control.
- Each column 6 well contains a dried-down IonCode™ barcode. The lowest barcode number is in A6, and the highest barcode number is in H6. All appear light blue in the actual plates.
- Wells A5 and B5 have 60 µL nuclease-free water. Water is added only if input DNA < 20 ng.


4. Add 60 µL of nuclease free water into wells A5 and B5 of the plate if using 10–20 ng high quality starting DNA. If you are using more than 20 ng of high quality starting DNA, you do not need to add 60 µL of nuclease free water into wells A5 and B5.

Note: If the mean read length is observed to be shorter than expected or shorter than the control, 60 µL of nuclease-free water needs to be added to wells A5 and B5. Low input and low-quality samples benefit from adding 60 µL of nuclease-free water to wells A5 and B5.

5. Carefully inspect each well for air bubbles. Remove any air bubbles by gentle pipetting. Alternatively, centrifuge the plate briefly in a plate centrifuge.
6. If you are processing fewer than 8 samples, it is preferable to add replicates or positive control samples to the run. Otherwise, pipet 15 µL of nuclease-free Water as non-template control into column 1 wells that do not contain a DNA sample.

Load the Ion Chef™ Instrument

IMPORTANT! When loading the instrument, do not force a cartridge into place. Each cartridge fits only one location on the deck and in one orientation. If a cartridge does not fit, verify that you are loading the correct cartridge in the correct orientation.

1. Touch  (Open Door) in the upper right corner of the touchscreen, wait for the door latch to open, then lift the door to the top of the travel until the latch engages.
2. Gently tap the Ion AmpliSeq™ Chef Reagents DL8 cartridge on the bench to force the reagents to the bottom of the tubes, then load the cartridge into the Reagents station so that it snaps into place.
3. Load an empty Tip Cartridge L8 from a previous run into the Used Pipette Tip station.
4. Load a new Ion AmpliSeq™ Tip Cartridge L8 into the New Pipette Tip station (left side of deck).
 - a. Unwrap the Ion AmpliSeq™ Tip Cartridge L8, then remove the cover to expose the pipette tips.
 - b. Slide the catch forward to allow the locking bracket to pivot upward. Load the Ion AmpliSeq™ Tip Cartridge L8 into position, pull the bracket downward, then push the catch backward to lock the cartridge in place.
5. Load the IonCode™ 96 Well PCR Plate containing gDNA onto the thermal cycler sample block, with position A1 in the upper left corner, then press down to seat it.
6. With the white dot on the PCR Plate Frame facing upward, load the PCR Plate Frame into the thermal cycler sample block pressing down firmly on each corner, then insert a new PCR Frame Seal underneath the automated heated cover. Ensure that the PCR Plate Frame is pressed completely down onto the thermal cycler block and that the PCR Plate Frame sits lower than the PCR Plate.

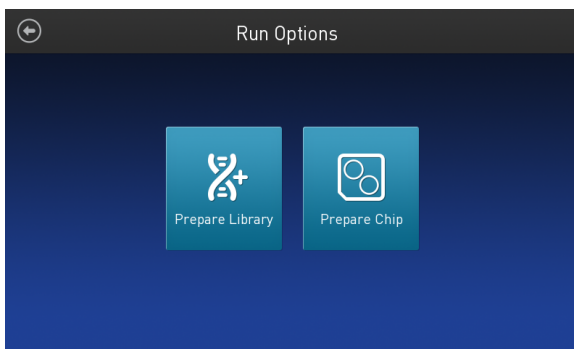
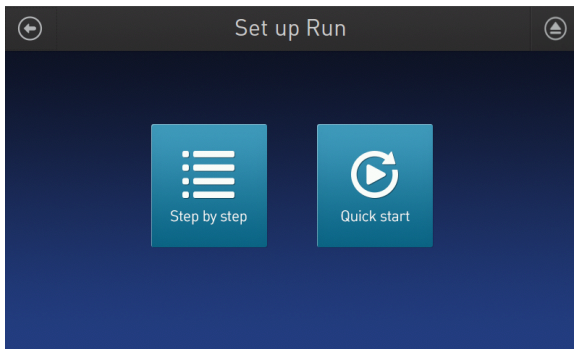
7. Load the Enrichment Cartridge into the Enrichment station.
8. Close the instrument door by first lifting it up slightly to disengage the locking mechanism, then pushing down on the door so that the lower locks engage.

Start the Ion Chef™ run

1. On the Ion Chef™ home touchscreen, touch **Set up run**.



2. Select one of the following actions.
 - Touch **Step by step**, then touch **Prepare Library** on the **Run Options** screen.
 - Ensure that you have loaded the instrument with all kits and consumables, touch **Quick start**, then proceed to step 4. Selecting **Quick start** bypasses the step by step deck loading steps.



3. (Step by step set up only) Ensure that you have completely loaded the Ion Chef™ deck with Ion AmpliSeq™ Kit for Chef DL8 consumables by advancing through the Step by Step deck loading steps on the instrument touch screen.
4. Touch **Start check** on the **Close Door** screen. The Ion Chef™ Instrument performs a Deck Scan.



Note: If the PCR plate is not recognized, select the appropriate plate when prompted. If no sample set was selected or planned in the Torrent Server, the following warning appears: "No sample Set detected. Do you want to continue?" Touch **OK**.

5. After Deck Scan completes (~3 minutes), touch **Next**.
6. On the Data Destination screen, ensure the Server and Sample set information, then touch **Next**.
7. Determine the number of amplicons for your panel.

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

8. Enter the appropriate number of primer pools, target amplification cycles, and an anneal/extension time for your run.

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	29	32
Other panels		
12–24	30	33
25–48	29	32
49–96	28	31
97–192	27	30
193–384	26	29
385–768	25	28

^[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.

Note: The Anneal/Extension time for all assays is 4 minutes.


The number of amplification cycles that are recommended in the preceding tables are based on 10–20 ng DNA input. Adjust the number of amplification cycles for lower or higher DNA input:

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

9. Touch **Start Run**.
10. After approximately 7 hours, return to the Ion Chef™ Instrument. On the **Run Complete** screen, touch **Next** to proceed to the unloading and cleaning steps.

IMPORTANT! The Ion Chef™ Instrument holds the barcoded libraries in the tube in Position D of the Reagents cartridge. Remove and cap the tube as soon as possible after run completion. Do not leave the tube in the instrument longer than 24 hours after the start of the run. After 24 hours from the start of the run, the instrument chiller stops actively cooling, and the sample is held at 27°C.

Unload the Ion Chef™ Instrument

1. Open the instrument door:
 - a. In the instrument touchscreen, touch  (Open Door), then wait for the latch to open.

- Lift the instrument door to the top of the travel until the latch mechanism engages.
- Remove the Ion AmpliSeq™ Chef Reagents DL8 cartridge. Remove and cap the combined library tube from Position D, then discard the cartridge.

IMPORTANT! If you are using the Oncomine™ Comprehensive Assay Plus – Automated Library Preparation, cap both combined library tubes from Positions C and D. The Oncomine™ Comprehensive Assay Plus – Automated Library Preparation combines 4 libraries per pool, A1–A4 in Position C and A5–A8 in position D.

- Remove, then discard the Ion AmpliSeq™ Chef Solutions DL8 cartridge.
 - Remove, then discard the IonCode™ 96 Well PCR Plate, PCR Plate Frame, and seal from the thermal cycler sample block in unison.
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- Remove, then discard the box of used pipette tips from the Used Pipette Tip station. Discard liquid waste in the tip box by pouring the waste into a waste container through the corner slot.
 - Move the empty Tip Cartridge L8 from the New Pipette Tip station to the Used Pipette Tip station.
 - Remove and discard the Enrichment Cartridge.

IMPORTANT! After completion of an Ion Chef™ run, clean the instrument. See the *Ion AmpliSeq™ Library Preparation on the Ion Chef™ System User Guide* (Pub. No. MAN0013432) for more information.

The libraries are at ~100 pM (total combined library concentration) and are ready to use in template preparation. Store unused portions of combined libraries at 4°C to 8°C for up to 1 month. For longer-term storage, store at –30°C to –10°C. See the appropriate Ion Chef™ or Ion OneTouch™ 2 template kit user guide for detailed instructions for template preparation.

Purify the libraries

- Remove the final library pool from instrument after the run has finished.
- Vortex the final library pool tube briefly, then centrifuge to collect the contents.
- Transfer 50 µL of the final library pool (library pool) to a new 1.5-mL Eppendorf™ LoBind™ tube or MicroAmp™ Optical 96-Well Reaction Plate.
- Add 50 µL (1X sample volume) of Agencourt™ AMPure™ XP Reagent to the library pool, then pipet up and down 5 times to thoroughly mix the bead suspension with the DNA.
- Incubate the mixture for 5 minutes at room temperature.
- Place the plate in a magnetic rack such as the DynaMag™-96 Side Magnet or the tube in a 1.5-mL magnet such as the DynaMag™-2 Magnet, then incubate for 5 minutes or until the solution clears.

7. Carefully remove, then discard the supernatant without disturbing the pellet.
8. 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.

Note: If your magnet does not have two positions for shifting the beads, remove the plate from the magnet and gently pipet up and down 5 times (with the pipettor set at 100 µL), then return the plate to the magnet and incubate for 2 minutes or until the solution clears.

9. Carefully remove, then discard the supernatant without disturbing the pellet.
10. Repeat step 8–9 for a second wash.
11. 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.

Elute the libraries

1. Remove the plate or tube with purified libraries from the magnet, then add 50 µL of Low TE to the pellet.
2. Seal the plate with MicroAmp™ Clear Adhesive Film or cap the tube, vortex thoroughly, then briefly centrifuge to collect contents.
3. Incubate at room temperature for at least 2 minutes.
4. Place the plate or tube on the magnet for at least 2 minutes.
5. Transfer ~48 µL of the library to a new 1.5-mL Eppendorf™ LoBind™ tube without disturbing the bead pellet.

STOPPING POINT Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at -20°C.

Assume that library concentration is 100 pM or check by following qPCR (see “Quantify library by qPCR and calculate the dilution factor” on page 25) with a 1:100 dilution (2 µL library + 198 µL Nuclease-free water).

About Planned Runs

Planned Runs are digital instructions that are created in Torrent Suite™ Software for controlling the template preparation and sequencing instruments. Planned Runs contain settings such as number of flows, kit types, barcodes, sample information, and reference files (if any). Planned Runs are also used to track samples, chips, and reagents throughout the workflow, from template preparation on the Ion Chef™ Instrument through sequencing on an and subsequent data analysis. Each chip that is prepared in an Ion Chef™ run requires its own Planned Run.

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


(continued)

Field name	Parameter
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
Library Kit Type	Leave blank
Barcode Set	Select the barcode set that was used for library preparation.
Template Kit	<ol style="list-style-type: none"> 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.

(continued)

Field name	Parameter
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.
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.

Run Plan Name (required) :

① — Plant_combo

② — **Analysis Parameters:** Default (Recommended) Custom Details +

Default Reference & BED Files —

Reference Library :

Target Regions:

Hotspot Regions:

③ — Use same reference & BED files for all barcodes

④ — **Number of barcodes :** Save Samples Table Load Samples Table

⑤ — **Sample Tube Label :**

⑥ — **Chip Barcode :**

Enter a sample name for each barcode used (require at least one sample) :

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 <input checked="" type="radio"/> 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.

#	Barcode	Sample (required)	Control Type	Sample ID	Sample Description	Reference Annotations
1	IonCode_0101 (CTAAGGTAAC) ▼	Sample 1				
2	IonCode_0102 (TAAGGAGAAC) ▼	Sample 2				
3	IonCode_0103 (AAGAGGATTC) ▼	Sample 3				
4	IonCode_0104 (TACCAAGATC) ▼	Sample 4				

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	<i>(Optional)</i> Click in the field, then enter a sample ID.
5	Sample Description	<i>(Optional)</i> 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)	<i>Ion 520™ & Ion 530™ ExT Kit – Chef User Guide</i> (Pub. No. MAN0015805)

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

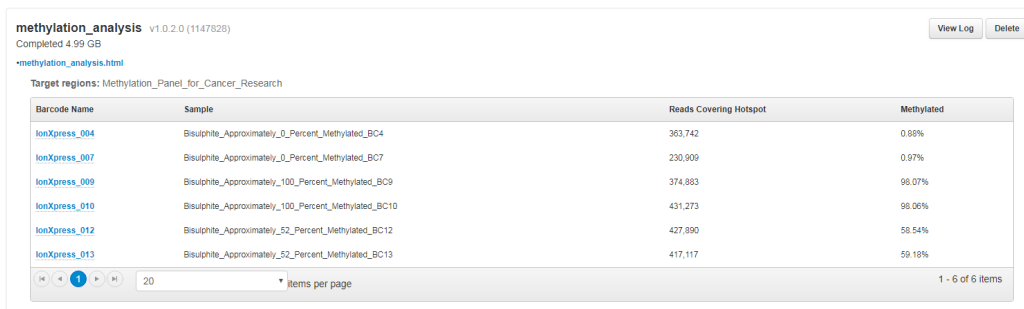
Note:

- 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.



The screenshot shows the 'methylation_analysis' plugin interface. At the top, it indicates 'v1.0.2.0 (1147828)' and 'Completed 4.99 GB'. There are 'View Log' and 'Delete' buttons. Below this, the target regions are listed as 'Methylation_Panel_for_Cancer_Research'. The main part of the interface is a table with the following data:

Barcode Name	Sample	Reads Covering Hotspot	Methylated
IonXpress_004	Bisulphite_Approximately_0_Percent_Methylated_BC4	363,742	0.88%
IonXpress_007	Bisulphite_Approximately_0_Percent_Methylated_BC7	230,909	0.97%
IonXpress_009	Bisulphite_Approximately_100_Percent_Methylated_BC9	374,883	98.07%
IonXpress_010	Bisulphite_Approximately_100_Percent_Methylated_BC10	431,273	98.06%
IonXpress_012	Bisulphite_Approximately_52_Percent_Methylated_BC12	427,890	58.54%
IonXpress_013	Bisulphite_Approximately_52_Percent_Methylated_BC13	417,117	59.18%

At the bottom of the table, there is a pagination control showing '20' items per page and '1 - 6 of 6 items'.

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.

- 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.

Bisulphite Methylation Analysis Report

Sample Name: Bisulphite_Approximately_0_Percent_Methylated_BC4

Run Name:

Reference: hg19_Lambda

Target regions: Methylation_Panel_for_Cancer_Research

^ File Links	
Download the target CpG Methylation Summary	?
Download the target CpG Methylation Detail	?
Download the insert CpG Methylation Summary	?
Download the insert CpG Methylation Detail	?
Download the insert Non CpG C Methylation Summary	?
Download the insert Non CpG C Methylation Detail	?
Download the bismark Log File	?
Download the bisulphite BAM	?

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.
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.

(continued)

File	Description
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](https://www.iongen.com/AmpliSeq.com)).

Supplemental procedures

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- μ L 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 μ L of 2X Ion Library qPCR Master Mix and 2 μ L of Ion Library TaqMan™ Quantitation Assay, 20X, then mix thoroughly. Dispense 11- μ L aliquots into the wells of a PCR plate.
3. Add 9 μ L of the diluted (1:100) Ion AmpliSeq™ library or 9 μ L of each control dilution to each well (two wells per sample as noted before), for a total reaction volume of 20 μ L.
4. Seal the plate with a MicroAmp™ Optical Adhesive Film, vortex thoroughly, then briefly centrifuge to collect droplets.
5. 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 μ L.
 - 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	Run mode	Stage	Temp	Time
7500 Fast	Fast	Hold ^[1]	50°C	2 min
7900 HT 7900 HT Fast		Hold ^[2]	95°C	20 sec
ViiA™ 7		Cycle (40 cycles)	95°C	1 sec
StepOne™ StepOnePlus™			60°C	20 sec
QuantStudio™ 3, 5, or 7				
7300		Standard	Hold ^[1]	50°C
7500	Hold ^[2]		95°C	2 min
7900 HT 7900 HT Fast	Cycle (40 cycles)		95°C	15 sec
ViiA™ 7			60°C	1 min
QuantStudio™ 3, 5, or 7				

^[1] UDG incubation

^[2] Polymerase activation

- Following qPCR, calculate the average concentration of the undiluted Ion AmpliSeq™ library by multiplying the concentration that is determined with qPCR by 100.
- Based on the calculated library concentration, determine the dilution that results in a concentration of ~50–100 pM.
- Dilute library to ~50–100 pM, combine, then proceed to template preparation, or store libraries as described below.

STOPPING POINT Libraries can be stored at 4–8°C for up to 1 month. For longer term, store at -20°C.

Clean the Ion Chef™ Instrument

About the cleaning protocol

The Ion Chef™ System includes an automated cleaning function that must be performed following every run. The cleaning routine is initiated from the Ion Chef™ Instrument touchscreen and is designed to minimize potential contamination. During the routine, the instrument irradiates the deck with ultraviolet light for 1 minute after all consumables have been removed from the instrument.

IMPORTANT! Although the Ion Chef™ Instrument cleaning routine provides some protection against contamination, it is not a substitute for good laboratory technique or precautions. When preparing DNA libraries for use or when preparing the Ion Chef™ Instrument, make certain to observe sterile laboratory procedures at all times to ensure minimal contamination.

Clean the Ion Chef™ Instrument

IMPORTANT! Clean the Ion Chef™ Instrument after every run. To prevent contamination, do not operate the instrument unless it has been recently cleaned.

1. Close the instrument door by first lifting it slightly to disengage the locking mechanism, then pushing down on the door until the locks engage.
2. On the Ion Chef™ Instrument touchscreen that appears after run completion, tap **Next**.
3. Ensure that you have removed all consumables from the Ion Chef™ Instrument, then tap **Next**.
4. With the door closed, tap **Start**.

The instrument performs a Deck Scan before starting the cleaning routine. The Ion Chef™ Instrument stops ventilation and illuminates the ultraviolet (UV) light in the instrument.



CAUTION! The Ion Chef™ Instrument emits UV light at 254 nm. Wear appropriate eye wear, protective clothing, and gloves when working near the instrument. Do not look directly at the UV light while it is illuminated during the cleaning routine.



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For descriptions of symbols on product labels or product documents, go to [thermofisher.com/symbols-definition](https://www.thermofisher.com/symbols-definition).

Revision history: MAN0017892 D.0 (English)

Revision	Date	Description
D.0	20 September 2023	<ul style="list-style-type: none">• Updated Ion AmpliSeq™ Chef Supplies DL8 (Part No. A29027) with the PCR Plate Frame.• Updated instructions to include loading the PCR Plate Frame. See “Load the Ion Chef™ Instrument” on page 11.• Updated instructions to include removing the PCR Plate Frame. See “Unload the Ion Chef™ Instrument” on page 14.
C.0	3 June 2019	<ul style="list-style-type: none">• Corrected Sequencing kit parameter in “Planned Run wizard key fields” on page 17.• Updated “Start the Ion Chef™ run” on page 12.
B.0	19 February 2019	Edit to figure in “Add DNA to the IonCode™ PCR plate” on page 10.
A.0	29 January 2019	New user bulletin for bisulfite methylation library production and analysis using the Ion AmpliSeq™ Kit for Chef DL8.

The information in this guide is subject to change without notice.

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