Chapter 20

Targeted Bisulfite Sequencing Using the SeqCap Epi Enrichment System

Jennifer Wendt, Heidi Rosenbaum, Todd A. Richmond, Jeffrey A. Jeddeloh, and Daniel L. Burgess

Abstract

Cytosine methylation has been shown to have a role in a host of biological processes. In mammalian biology these include stem cell differentiation, embryonic development, genomic imprinting, inflammation, and silencing of transposable elements. Given the central importance of these processes, it is not surprising to find aberrant cytosine methylation patterns associated with many disorders in humans, including cancer, cardiovascular disease, and neurological disease. While whole genome shotgun bisulfite sequencing (WGBS) has recently become feasible, generating high sequence coverage data for the entire genome is expensive, both in terms of money and analysis time, when generally only a small subset of the genome is of interest to most researchers. This report details a procedure for the targeted enrichment of bisulfite treated DNA via SeqCap Epi, allowing high resolution focus of next generation sequencing onto a subset of the genome for high resolution cytosine methylation analysis. Regions ranging in size from only a few kb up to over 200 Mb may be targeted, including the use of the SeqCap Epi CpGiant design which is designed to target 5.5 million CpGs in the human genome. Finally, multiple samples may be multiplexed and sequenced together to provide an inexpensive method of generating methylation data for a large number of samples in a high throughput fashion.

Key words Sequencing, NGS, Bisulfite, Targeted enrichment, SeqCap Epi, Hybridization

1 Introduction

The most commonly studied post-replicative and reversible modification of DNA is cytosine methylation [1]. Cytosine methylation has been shown to have a role in a host of biological processes. In mammalian biology these include stem cell differentiation, embryonic development, genomic imprinting, inflammation, and silencing of transposable elements. Given the central importance of these processes, it is not surprising to find aberrant cytosine methylation patterns associated with many disorders in humans, including cancer, cardiovascular disease, and neurological disease. In plant biology, cytosine methylation has been found to be similarly

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important for transposable element and gene silencing, as well as for genome stability. Recent interest among plant biologists in cytosine methylation comes from the commercialization of transgenic crop species since the transgene function can be susceptible to the methylation patterns of DNA adjacent to the integration site. In both plant and animal genomes an understanding of cytosine methylation patterns ("DNA methylation" hereafter) can be essential for understanding gene regulation. DNA methylation patterns are mitotically and meiotically stable, allowing them to be passed both to daughter cells and through the germ line to progeny. The ability to transmit DNA methylation patterns faithfully through multiple cellular generations, and the ability of the daughter cells to consistently interpret the pattern biologically, has led to their exploration as an inherited mechanism of gene regulation. Though heritable, DNA methylation is also reversible and generally considered an "epigenetic" mechanism of gene regulation, that is, regulation imparted "above" that of the nucleotide base sequence itself.

Since the development of robust methods for chemically deaminating cytosine with bisulfite were first published [2] analysis has increasingly been aimed at deciphering the methylation status of individual cytosine bases. Because the fidelity of cytosine methylation pattern maintenance is lower than the fidelity of the intrinsic base sequence [3], careful analysis requires a high depth of coverage to quantify the proportion of methylation occupancy at each cytosine position [4].

Whole genome shotgun bisulfite sequencing (WGBS) has recently become practical to consider as a means to characterize cytosine methylation patterns, as it provides DNA methylation data at single base resolution and allows for the assessment of the percentage of DNA methylation at each position in the genome by analyzing the same position across a population of individual molecules [5]. However, WGBS is time-consuming and costly due to a large amount of sequencing and data analysis required (most vertebrate and plant genomes contain hundreds of millions of cytosines), when generally 65% of the reads do not contain methylate-able sites (predominantly CG or CHG sites), and only a small subset of the genome is of interest to most researchers.

Chemical deamination of cytosine (C) bases to uracil (U) bases with bisulfite treatment brings a series of challenges to variant analysis, since millions of single nucleotide variants (SNVs) are created relative to the reference sequence. Each C base may be converted into a U base dependent upon whether or not it was methylated at the carbon-5 position (5mC). 5mC is substantially resistant to chemical deamination via bisulfite, while unmodified C is not. The U bases introduced by bisulfite are replaced with thymidine (T) bases during conventional DNA amplification and the sequence is analyzed for C or T content at C positions in the reference. The appearance of a T at a particular cytosine location

in the reference is interpreted as evidence that the original C was not methylated in the sample. Bisulfite conversion chemistry is effectively chemical mutagenesis ex vivo. Substantial analytical complexity is imposed by converting a large fraction of C bases in a genome, and this happens on two levels. First, bisulfite conversion effective doubles a haploid genome by making the DNA strands noncomplementary to each other. Second, the loss of many Cs from the previously well-balanced four base repertoire of DNA reduces the algorithmic complexity of the new larger genome. Bioinformatic analyses of bisulfite treated DNA sequence including mapping and assembly routines, must take into account that each apparent C or T might have started as a C, a 5mC, or even a T polymorphism relative to the reference, and then analyze the variants across all molecules spanning the same position as a way to characterize the average methylation occupancy of the cellular population.

Whole genome sequencing with current instruments requires that a sequencing library be generated from a DNA sample. A series of laboratory manipulations fragments the DNA, repairs damaged ends, and ligates sequencing adapters onto them. The libraries are often then amplified before they are sequenced. The subsequent data analysis provides a comprehensive genome sequence by ensuring that each base in a genome is represented on multiple molecules in the library and then repeatedly sampling that library. However, only a fraction of the genome is typically of interest to most researchers. Sequence Capture [6] technology provides a means to focus the depth of coverage available with current sequencing systems over genomic regions of interest [7]. This is accomplished by hybridizing a sequencing library to immobilized biotinylated oligonucleotide probes, which are designed to be complementary to the regions of interest in a genome. Library fragments that are not complementary to probes can be washed away, leaving the regions of interest over-represented in the resulting captured library.

To integrate Sequence Capture technology with a bisulfite sequencing approach, at least two workflows are possible. A universal constraint on both workflows is that, since the methylation patterns on DNA molecules recovered from a biological sample are not copied with currently available DNA amplification systems, any necessary amplification steps must only occur after bisulfite treatment of the DNA sample is completed. In the first workflow, a sequencing library is constructed, then library fragments from the regions of interest are captured, bisulfite treated, amplified and sequenced. In the second workflow, a sequencing library is constructed, then bisulfite treated *before* the fragments of interest are captured, amplified and sequenced (Fig. 1).

The first workflow (capture then convert) has the advantage of allowing the capture probes to be designed in native (four base)

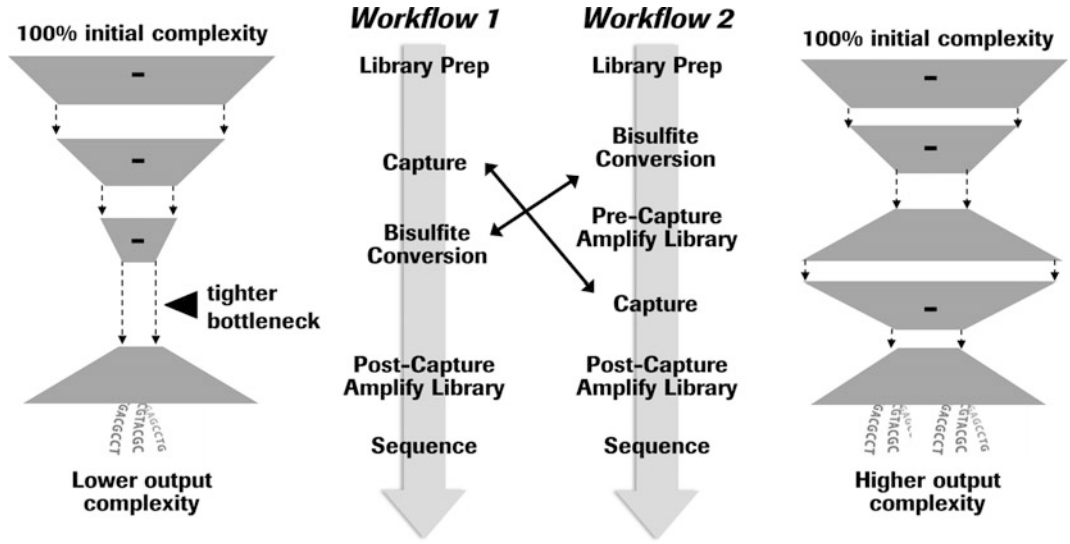

Fig. 1 Alternate workflows for integrating Sequence Capture and bisulfite sequencing. Library preparation, bisulfite conversion, and sequence capture are all steps where significant molecular complexity and information can be lost through inefficiency or bias. If those three steps are arranged in an uninterrupted series, as shown for Workflow 1, the effects are compounded and the information complexity bottleneck is tighter. More sample is required to offset the bottleneck effect and a higher rate of duplicate sequencing reads is expected. In Workflow 2 (SeqCap Epi), the three steps are arranged so that an intervening pre-capture amplification increases the total mass of DNA prior to the capture step to alleviate the bottleneck effect

sequence space, which requires fewer probes and should allow for higher capture specificity manifest as a higher on-target read rate. This method suffers from a hidden disadvantage, however, namely that the small molecular population recovered from capture of the sample library must be bisulfite converted before it can be amplified and sequenced. Bisulfite conversion is a harsh process that can damage as much as 90% of the library fragments to the extent that they cannot be amplified. Chaining each of these low yield steps together increases the potential that output sequencing libraries will suffer from low molecular diversity. Sequencing of a low diversity library yields highly redundant and more readily biased data, which may not be reflective of the actual methylation pattern in the sample.

The second capture workflow (convert then capture) does not suffer the same limitation, since an amplification step prior to capture is possible, which can mitigate diversity losses without affecting the integrity of the methylation pattern on each molecule. However, workflow 2 does have a distinct disadvantage relative to workflow 1 in terms of probe design; since the methylation occupancy of the relevant Cs are not known prior to capture, the probe design must take into account the myriad of possible methylation states for each C in each probe target. The practical effect of this disadvantage is that probe design and synthesis must address a

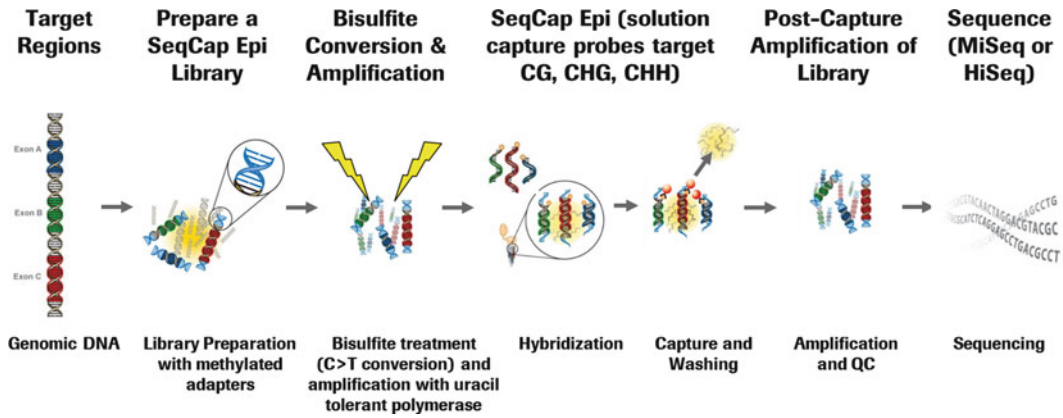

Fig. 2 SeqCap Epi workflow. The SeqCap Epi workflow incorporates bisulfite treatment and amplification steps before the hybrid capture step to reduce information loss through compounded molecular bottleneck effects

much larger possible sequence space to ensure high efficiency of molecule recovery in a manner irrespective of the actual native methylation occupancy of each molecule.

The SeqCap Epi Enrichment System, employing workflow 2, has overcome these challenges by utilizing a design algorithm that creates probes complementary to the possible methylation configurations on both strands from a bisulfite converted genomic template, and a manufacturing process which can create DNA oligonucleotide probe pools with tens of millions of oligos. This manufacturing process and the innovations in probe design have made the second workflow (convert then capture; Fig. 2) not only technically feasible, but effective. The SeqCap Epi Enrichment system enables the reproducible targeting of selected genomic regions, up to 210 Mb in cumulative size, from bisulfite treated genomic DNA, in a single workflow. The system has been validated in both plant (maize) and mammalian genomes (mouse and human) [8–10].

2 Materials

2.1 DNA Sample Library Preparation and Bisulfite Conversion

1. KAPA Library Prep Kit Illumina (Roche) or similar.
2. TE Buffer (1×): 10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA.
3. SeqCap Adapter Kit A (Roche).
4. SeqCap Adapter Kit B (Roche).
5. Agencourt AMPure XP (Beckman Coulter).
6. Ethanol, 200 proof (absolute), for molecular biology.
7. EZ DNA Methylation-Lightning Kit (Zymo Research or similar).

2.2 SeqCap Epi Enrichment System

1. SeqCap Epi CpGiant Enrichment Kit, SeqCap Epi Choice, or SeqCap Epi Developer (Roche). This kit contains the enrichment probe pool.
2. PCR Grade Water.
3. SeqCap EZ Hybridization and Wash Kit (Roche). This kit contains Bead Wash Buffer, Stringent Wash Buffer, Wash Buffer I, Wash Buffer II, Wash Buffer III, 2× Hybridization Buffer, and Component A.
4. SeqCap Epi Accessory Kit (Roche); This kit contains the Kapa HiFi HotStart ReadyMix, PCR Grade Water, Post-capture LM-PCR oligos, Bisulfite Conversion Control, and Bisulfite Capture Enhancer.
5. SeqCap HE-Oligo Kit A (Roche).
6. SeqCap HE-Oligo Kit B (Roche).
7. SeqCap EZ Pure Capture Bead Kit (Roche). This kit contains Purification Beads and Capture Beads.
8. High Sensitivity DNA Kit (Agilent).
9. DNA 1000 Kit (Agilent).

3 Methods

3.1 Storing the SeqCap Epi Probe Pool, Accessory Kit, Adapter Kits A and B, HE-Oligo Kits A and B, and Pure Capture Bead Kits

1. Upon receipt, undertake the following steps to ensure the highest performance of the SeqCap Epi probe pool to avoid multiple freeze/thaw cycles or potential accidental contamination:
   (a) If frozen, thaw the tube of SeqCap Epi probe pool on ice.
   (b) Vortex the SeqCap Epi probe pool for 3 s.
   (c) Centrifuge the tube of SeqCap Epi probe pool at 10,000 × *g* for 30 s to ensure that the liquid is at the bottom of the tube before opening.
   (d) Aliquot the SeqCap Epi probe pool into single-use aliquots (4.5 μL/aliquot) in 0.2 mL PCR tubes (or 96-well plates if following the higher throughput protocol) and store at −15 to −25 °C until use. The presence of some residual volume after dispensing all single-use aliquots is normal.
   (e) When ready to perform the experiment, thaw the required number of single-use SeqCap Epi probe pool aliquots on ice.
2. Upon receipt, store the SeqCap Epi Accessory Kit, SeqCap Hybridization and Wash Kit, SeqCap Adapter Kits A and B, and SeqCap HE-Oligo Kits at −15 to −25 °C.
3. Upon receipt, store the SeqCap Pure Capture Bead Kit at +2 to +8 °C.

3.2 Preparing the Sample Library and Performing Bisulfite Conversion

1. Resuspend the SeqCap adapters.
   (a) Spin the lyophilized index adapters, contained in the SeqCap Adapter Kit A and B, briefly to allow the contents to pellet at the bottom of the tube.
   (b) Add 50 μL cold, PCR-grade water to each of the 12 tubes labeled "SeqCap Index Adapter" in the SeqCap Adapter Kit A or B. Keep adapters on ice.
   (c) Briefly vortex the index adapters plus PCR-grade water and spin down the resuspended index adapter tubes.
   (d) The resuspended index adapter tubes should be stored at −15 to −25 °C.
2. Preparation of the bisulfite conversion control.
   (a) Briefly spin down the tube containing the Bisulfite conversion control to ensure that the entire tube contents are in the bottom of the tube.
   (b) Add 1 mL of PCR-grade water directly to the tube containing the Bisulfite conversion control.
   (c) Vortex for 10 s to mix.
   (d) Briefly spin down.
   (e) Aliquot 990 μL of the PCR-grade water into a new 1.5 mL tube.
   (f) Add 10 μL of the diluted Bisulfite conversion control to the tube containing 990 μL of PCR-grade water.
   (g) Vortex for 10 s to mix.
   (h) Briefly spin down. The Bisulfite conversion control is now ready for use. The diluted Bisulfite conversion control should be stored at −15 to −25 °C and repeated freeze/thaws should be avoided by creating aliquots of this diluted reagent.
3. Preparing the sample library.
   (a) Add 5.8 μL of the diluted Bisulfite conversion control to 1 μg of the gDNA sample of interest.
   (b) Vortex for 10 s to mix.
   (c) Adjust the volume of the combined gDNA sample and Bisulfite conversion control to a total volume of 52.5 μL using 1× TE and transfer to a Covaris microTUBE for fragmentation.
   (d) Fragment the DNA pool so that the average DNA fragment size is 180–220 bp.
   (e) Following fragmentation, transfer 50 μL of the fragmented DNA to a 0.2 mL PCR tube and construct the sample library following the procedure described in the KAPA

Library Preparation Kit Technical Data Sheet, v1.14 (or later).

- Follow the protocol beginning with "End Repair Reaction Setup" and "End Repair Cleanup", **steps 2** and **3** of the KAPA Library Preparation Kit Technical Data Sheet.
- Proceed to "A-Tailing Reaction Setup" and "A-Tailing Cleanup", **steps 4** and **5** of the KAPA Library Preparation Kit Technical Data Sheet.
- Proceed to "Adapter Ligation Reaction Setup", **step 6** of the KAPA Library Preparation Kit Technical Data Sheet. Use 5 μL of a 10 μM solution of the SeqCap Library Adapter of choice in the "Adapter Ligation Reaction Setup".
- Proceed to "Adapter Ligation Cleanup", **step 7** of the KAPA Library Preparation Kit Technical Data Sheet, and perform the "First Post-Ligation Cleanup". Skip the "Second Post-Ligation Cleanup".
- Proceed to the "Dual-SPRI Size Selection", **step 8** of the KAPA Library Preparation Kit Technical Data Sheet.

(f) Once the sample library is constructed, proceed with the bisulfite conversion.

4. Bisulfite conversion of the sample library.
   (a) Follow the steps in the Zymo Research "EZ DNA Methylation-Lightning Kit" for the bisulfite conversion of the DNA sample libraries.
   - Note: Some thermal cycles have a maximum volume capacity of 100 μL. When using a thermal cycler that is not designed to work with volumes greater than 100 μL, split the bisulfite conversion reaction into two separate 0.2 mL PCR tubes (75 μL per tube). Once the thermal cycler program has completed, the two-like samples can be purified using a single Zymo EZ DNA Methylation-Lightning Kit column.
   - Elute the purified bisulfite-converted sample library using 20 μL of PCR-grade water.

   (b) Once the bisulfite conversion of the DNA sample library is complete, proceed with amplification of the sample.

3.3 Amplifying the Bisulfite-Converted Sample Library Using LM-PCR

1. Resuspend the SeqCap Pre-LM-PCR oligos.
   (a) Spin the lyophilized primers briefly to allow the contents to pellet at the bottom of the tube. Please note that both primers are contained within a single tube.

(b) Add 550 μL PCR-grade water to the tube labeled "Pre-LM-PCR Oligos 1 & 2" from the SeqCap Adapter Kit A or B.

(c) Briefly vortex the primers plus PCR-grade water and spin down the resuspended oligo tube.

(d) The resuspended oligo tube should be stored at −15 to −25 °C.

2. Preparing the LM-PCR reaction.

(a) Prepare the LM-PCR Master Mix in a 1.5 mL microcentrifuge tube (or 15 mL conical) tube on ice. The amount of each reagent needed is listed below (if desired, increase all Master Mix volumes by 10% to account for pipetting variance). It is recommended to include both a negative (water) and positive (previously amplified library) controls in this step.

Pre-capture LM-PCR master mix	Per individual sample library or negative control (μL)	24 Sample libraries (μL)	96 Sample libraries (μL)
KAPA HiFi HotStart Uracil + ReadyMix (2×)[a]	25	600	2400
PCR grade water	2	48	192
Pre LM-PCR Oligo 1 and 2, 5 μM	3	72	288
Total	30	720	2880

[a]Note: The Kapa HiFi HotStart Uracil + Ready Mix (2×) is contained within the SeqCap Epi accessory kit

(b) Pipette 30 μL of LM-PCR Master Mix into each PCR tube or well.

(c) Add the 20 μL of the bisulfite-converted sample library (or PCR grade water for negative control) to the PCR tube, or each well of the 96-well plate, containing the LM-PCR Master Mix. Mix well by pipetting up and down five times. Do not vortex.

3. Performing the PCR amplification.

(a) Place the PCR tube (or 96-well PCR plate) in a thermal cycler.

(b) Amplify the bisulfite-converted sample library using the following Pre-Capture LM-PCR program: 2 min at 95 °C, followed by 12 cycles of 30 s at 98 °C, 30 s at 60 °C and 4 min at 72 °C, followed by 10 min at 72 °C, hold at 4 °C (*see* **Note 1**).

(c) Store the reaction at +2 to +8 °C (for up to 72 h) until ready for cleanup.

4. Purifying the amplified bisulfite-converted sample library using DNA purification beads (*see* **Note 2**).
   (a) Allow the DNA Purification Beads to warm to room temperature for at least 30 min prior to use.
   (b) Transfer each amplified, bisulfite-converted sample library into a separate 1.5 mL microcentrifuge tube (approximately 50 μL). Process the negative control in exactly the same way as the amplified, bisulfite-converted sample library.
   (c) Vortex the beads for 10 s before use to ensure a homogenous mixture of beads.
   (d) Add 90 μL (1.8× volume) DNA Purification Beads to the 50 μL of amplified, bisulfite-converted sample library.
   (e) Vortex briefly and incubate at room temperature for 15 min to allow the DNA to bind to the beads.
   (f) Place the tube containing the bead bound DNA in a magnetic particle collector.
   (g) Allow the solution to clear.
   (h) Once clear, remove and discard the supernatant being careful not to disturb the beads.
   (i) Add 200 μL of freshly prepared 80% ethanol to the tube containing the beads plus DNA. The tube should be left in the magnetic particle collector during this step.
   (j) Incubate at room temperature for 30 s.
   (k) Remove and discard the 80% ethanol and repeat **steps i–j**. for a total of two washes with 80% ethanol.
   (l) Following the second wash, remove and discard all of the 80% ethanol.
   (m) Allow the beads to dry at room temperature with the tube lid open for 15 min (or until dry) (*see* **Note 3**).
   (n) Remove the tube from the magnetic particle collector and resuspend the DNA using 52 μL of PCR-grade water (*see* **Note 4**).
   (o) Vortex for 10 s to mix to ensure that all of the beads are resuspended.
   (p) Incubate at room temperature for 2 min.
   (q) Place the tube back in the magnetic particle collector and allow the solution to clear.
   (r) Remove 50 μL of the supernatant that now contains the amplified, bisulfite-converted sample library and aliquot into a new 1.5 mL tube.

5. Checking the quality of the amplified, bisulfite-converted sample library.
   (a) Measure the A_{260}/A_{280} ratio of the amplified, bisulfite-converted sample library to quantify the DNA concentration using a NanoDrop spectrophotometer and determine the DNA quality.
      - The A_{260}/A_{280} ratio should be 1.7–2.0.
      - The sample library yield should be ≥1.0 μg.
      - The negative control yield should be negligible. If this is not the case, the measurement may be high due to the presence of unincorporated primers carried over from the LM-PCR reaction and not an indication of possible contamination between amplified sample libraries; however, this should be verified using an 2100 Bioanalyzer instrument as follows.
   (b) Run 1 μL of each amplified, bisulfite-converted sample library (and negative/positive controls) on a High Sensitivity DNA chip (Fig. 3). Run the chip according to the manufacturer's instructions. If the amplified, bisulfite-converted sample library has a concentration above 20 ng/μL consider diluting the sample 1:10 using PCR-grade water prior to running it on the High Sensitivity DNA chip. The Bioanalyzer trace should indicate that the average DNA fragment size falls between 150 and 500 bp. The negative control should not show any significant signal within this range, which could indicate

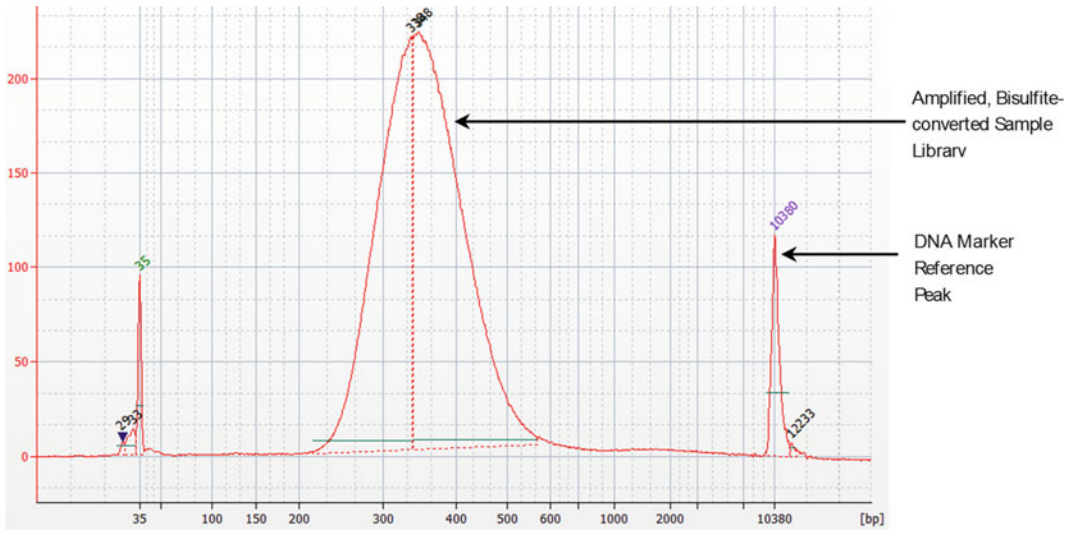

Fig. 3 Pre-Capture LM-PCR. Example of an amplified, bisulfite-converted sample library analyzed using an Agilent high sensitivity DNA chip

contamination between amplified sample libraries, but it may exhibit sharp peaks visible below 150 bp. If the negative control reaction shows a positive signal by NanoDrop spectrophotometer, but the Bioanalyzer trace indicates only the presence of a sharp peak below 150 bp in size, then the negative control should not be considered contaminated.

(c) If the amplified, bisulfite-converted sample library meets these requirements, proceed to hybridization. If the amplified, bisulfite-converted sample library does not meet these requirements, reconstruct the sample library.

3.4 Hybridizing the Amplified, Bisulfite-Converted Sample Library and SeqCap Epi Probe Pool

1. Preparing for hybridization.
   (a) Turn on a heat block to +95 °C and let it equilibrate to the set temperature.
   (b) Remove the appropriate number of 4.5 μL SeqCap Epi probe pool aliquots (one per bisulfite-converted sample library) from the −15 to −25 °C freezer and allow them to thaw on ice.
2. Resuspend the SeqCap HE universal and SeqCap HE Index oligos.
   (a) Spin the lyophilized oligo tubes, contained in the SeqCap HE-Oligo Kits A or B, briefly to allow the contents to pellet to the bottom of the tube.
   (b) Add 120 μL PCR-grade water to the SeqCap HE Universal Oligo tube (1000 μM final concentration).
   (c) Add 10 μL PCR-grade water to each SeqCap HE Index Oligo tube (1000 μM final concentration).
   (d) Vortex the primers plus PCR-grade water for 5 s and spin down the resuspended oligo tube.
   (e) The resuspended oligo tube should be stored at −15 to −25 °C (*see* **Note 5**).
3. Prepare the amplified, bisulfite-converted DNA sample library and HE oligos for hybridization.
   (a) Thaw on ice the amplified, bisulfite-converted DNA sample library that will be used in the capture experiment.
   (b) One microgram of the amplified, bisulfite-converted DNA sample will be used in the sequence capture hybridization step.
   (c) Thaw on ice the resuspended SeqCap HE Universal Oligo (1000 μM) and the resuspended SeqCap HE Index Oligo (1000 μM) that matches the DNA Adapter Index in the amplified, bisulfite-converted DNA sample library.

4. Prepare the hybridization sample.
   (a) Add 10 μL of Bisulfite Capture Enhancer (of the SeqCap Epi Accessory Kit) to a new 1.5 mL tube.
   (b) Add 1 μg of the amplified, bisulfite-converted DNA sample library to the 1.5 mL tube containing the 10 μL aliquot of Bisulfite Capture Enhancer.
   (c) Add 1 μL of the SeqCap HE Universal Oligo and 1 μL of the appropriate SeqCap HE Index Oligo to the amplified, bisulfite-converted DNA sample plus Bisulfite Capture Enhancer.
   (d) Close the tube's lid and make a hole in the top of the tube's cap with an 18–20 G (or smaller) needle. The closed lid with a hole in the top of the tube's cap is a precaution to suppress contamination in the DNA vacuum concentrator.
   (e) Dry the amplified, bisulfite-converted DNA sample/Bisulfite Capture Enhancer/Hybridization Enhancing Oligos in a DNA vacuum concentrator on high heat (+60 °C) (*see* **Note 6**).
      - To each dried-down amplified, bisulfite-converted DNA sample/Bisulfite Capture Enhancer/Hybridization Enhancing Oligos, add 7.5 μL of 2× Hybridization Buffer and 3 μL of Hybridization Component A (*see* **Note 7**).
      - The tube with the amplified, bisulfite-converted DNA sample/Bisulfite Capture Enhancer/Hybridization Enhancing Oligos should now contain the following components:

Component	Solution capture
Bisulfite capture enhancer	100 μg
Amplified, bisulfite-converted DNA sample	1 μg
Hybridization enhancing Oligos	2000 pmol[a]
2X hybridization buffer	7.5 μL
Hybridization component A	3 μL
Total	*10.5 μL*

[a]Composed of 50% (1000 pmol) SeqCap HE Universal Oligo and 50% (1000 pmol) of the appropriate SeqCap HE Index oligo

- Cover the hole in the tube's cap with a sticker or small piece of laboratory tape.
- Vortex the amplified, bisulfite-converted DNA sample/Bisulfite Capture Enhancer/Hybridization Enhancing Oligos plus

Hybridization Cocktail (2× Hybridization Buffer + Hybridization Component A) for 10 s and centrifuge at maximum speed for 10 s.

- Place the amplified, bisulfite-converted DNA sample/Bisulfite Capture Enhancer/Hybridization Enhancing Oligos/Hybridization Cocktail in a + 95 °C heat block for 10 min to denature the DNA.
- Centrifuge the amplified, bisulfite-converted DNA sample/Bisulfite Capture Enhancer/Hybridization Enhancing Oligos/Hybridization Cocktail at maximum speed for 10 s at room temperature.
- Transfer the amplified, bisulfite-converted DNA sample/Bisulfite Capture Enhancer/Hybridization Enhancing Oligos/Hybridization Cocktail to the 4.5 μL aliquot of SeqCap Epi probe pool in a 0.2 mL PCR tube (the entire volume can also be transferred to one well of a 96-well PCR plate).
- Vortex for 3 s and centrifuge at maximum speed for 10 s.
- The hybridization sample should now contain the following components:

Component	Solution capture
Bisulfite capture enhancer	100 μg
Amplified, bisulfite-converted DNA sample	1 μg
Hybridization enhancing Oligos	2000 pmol[a]
2× hybridization buffer	7.5 μL
Hybridization component A	3 μL
SeqCap Epi probe pool	4.5 μL
Total	*15 μL*

[a]Composed of 50% (1000 pmol) SeqCap HE Universal Oligo and 50% (1000 pmol) of the appropriate SeqCap HE Index Oligo

- Incubate in a thermal cycler at +47 °C for 64–72 h. The thermal cycler's heated lid should be turned on and set to maintain +57 °C (10 °C above the hybridization temperature).

3.5 Washing and Recovering the Captured Bisulfite-Converted DNA Sample

1. Preparing sequence capture and bead wash buffers.
   (a) Dilute the 10× Wash Buffer (I, II, III, and Stringent) and the 2.5× Bead Wash Buffer to create 1× working solutions (all of the buffers can be found in the SeqCap Hybridization and Wash Kit).

Amount of concentrated buffer	Amount of PCR grade water (μL)	Total volume of 1× buffer[a] (μL)
40 μL—10× stringent wash buffer	360	400
30 μL—10× wash buffer I	270	300
20 μL—10× wash buffer II	180	200
20 μL—10× wash buffer III	180	200
200 μL—2.5× bead wash buffer	300	500

[a]Store working solutions at room temperature (+15 to +25 °C) for up to 2 weeks. The volumes in this table are calculated for a single experiment; scale up accordingly if multiple samples will be processed

(b) Preheat the following wash buffers to +47 °C in a water bath:
- 400 μL of 1× Stringent Wash Buffer.
- 100 μL of 1× Wash Buffer I.

2. Preparing the capture beads.
   (a) All the Capture Beads to warm to room temperature for 30 min prior to use.
   (b) Mix the beads thoroughly by vortexing for 15 s.
   (c) Aliquot 100 μL of bead for each capture into a single 1.5 mL tube (i.e., for one capture use 100 μL of beads and for four captures using 400 μL of beads, etc.).
   (d) Place the tube in a magnetic particle collector. When the liquid becomes clear, remove and discard the liquid being careful to leave all of the beads in the tube. Any remaining traces of liquid will be removed wit subsequent wash steps.
   (e) While the tube is in the magnetic particle collector, add twice the initial volume of beads of 1× Bead Wash Buffer (i.e., for one capture use 200 μL of 1× Bead Wash Buffer and for four captures use 800 μL of 1× Bead Wash Buffer, etc.).
   (f) Remove the tube from the magnetic particle collector and vortex for 10 s.
   (g) Place the tube back in the magnetic particle collector to bind the beads. Once clear, remove and discard the liquid.
   (h) Repeat **steps e–g** for a total of two washes.
   (i) After removing the buffer following the second wash, resuspend by vortexing the beads in 1× the original volume using 1× Bead Wash Buffer (i.e., for one capture using 100 μL 1× Bead Wash Buffer and for four captures use 400 μL 1× Bead Wash Buffer, etc.).

(j) Aliquot 100 μL of resuspended beads into new 0.2 mL tubes (one tube per captured sample).

(k) Place the tube in the magnetic particle collector to bind the beads. Once clear, remove and discard the liquid.

(l) The Capture Beads are now ready to bind the captured DNA. Proceed immediately to "**step 3**" (below).

3. Binding DNA to the capture beads.

(a) Transfer the hybridization samples to the tubes containing the Capture Beads prepared above.

(b) Mix thoroughly by pipetting up and down ten times.

(c) Bind the captured sample to the beads by placing the tubes containing the beads and DNA in a thermal cycler at +47 °C for 45 min (heated lid set to +57 °C). Mix the samples by vortexing for 3 s at 15 min intervals to ensure that the beads remaining in suspension. It is helpful to have a vortex mixer located close to the thermal cycler for this step.

4. Washing the capture beads plus bound DNA.

(a) After the 45-min incubation, add 100 μL of 1× Wash Buffer I heated to +47 °C to the 15 μL of Capture Beads plus bound DNA.

(b) Mix by vortexing for 10 s.

(c) Transfer the entire content of each 0.2 mL tube to 1.5 mL tubes.

(d) Place the tubes in a magnetic particle collector to bind the beads. Remove and discard the liquid once clear.

(e) Remove the tubes from the magnetic particle collector and add 200 μL of 1× Stringent Wash Buffer heated to +47 °C. Pipette up and down ten times to mix. Work quickly so that the temperature does not drop much below +47 °C.

(f) Incubate at +47 °C for 5 min.

(g) Repeat **steps d–f** for a total of two washes using 1× Stringent Wash Buffer heated to +47 °C.

(h) Place the tubes in the magnetic particle collector to bind the beads. Remove and discard the liquid once clear.

(i) Add 200 μL of room temperature 1× Wash Buffer I and mix by vortexing (continually) for 2 min. If liquid has collected in the tube's cap, tap the tube gently to collect the liquid into the tube's bottom before continuing to the next step.

(j) Place the tubes in the magnetic particle collector to bind the beads. Remove and discard the liquid once clear.

(k) Add 200 μL of room temperature 1× Wash Buffer II and mix by vortexing (continually) for 1 min.

(l) Place the tubes in the magnetic particle collector to bind the beads. Remove and discard the liquid once clear.

(m) Add 200 μL of room temperature 1× Wash Buffer III and mix by vortexing (continually) for 30 s.

(n) Place the tubes in the magnetic particle collector to bind the beads. Remove and discard the liquid once clear.

(o) Remove the tubes from the magnetic particle collector and add 50 μL of PCR-grade water to each tube of bead-bound captured DNA sample.

(p) Store the beads plus captured samples at −15 to −25 °C or proceed with the next step (*see* **Note 8**).

3.6 Amplifying the Captured, Bisulfite-Converted DNA Sample Using LM-PCR and Sequencing the Captured Samples

1. Resuspend the post-LM-PCR oligos.
   (a) Spin the lyophilized oligos, contained in the SeqCap Epi Accessory Kit, briefly to allow the contents to pellet at the bottom of the tube. Please note that both oligos are contained within a single tube.
   (b) Add 480 μL PCR-grade water to the tube labeled "Post-LM-PCR Oligos 1 & 2" from the SeqCap Epi Accessory Kit.
   (c) Briefly vortex the primers plus PCR-grade water and spin down the resuspended oligo tube.
   (d) The resuspended oligo tube should be stored at −15 to −25 °C.
2. Preparing the LM-PCR reaction.
   (a) Prepare the LM-PCR Master Mix in a 1.5 mL microcentrifuge tube (or 15 mL conical) tube on ice. The amount of each reagent needed for two reactions (one captured DNA sample) is listed below (if desired, increase all Master Mix volumes by 10% to account for pipetting variance). It is recommended to include both a negative (water) and positive (previously amplified library) controls in this step.

Pre-capture LM-PCR master mix	Two reactions (for one captured DNA sample)
KAPA HiFi HotStart ReadyMix (2×)[a]	50 μL
Post LM-PCR Oligo 1 & 2, 5 μM	10 μL
Total	*30 μL*

[a]Note: The Kapa HiFi HotStart Ready Mix (2×) is contained within the SeqCap Epi accessory kit

(b) Pipette 30 μL of LM-PCR Master Mix into the PCR tubes or wells.

(c) Vortex the bead-bound captured DNA to ensure a homogenous mixture of beads.

(d) Aliquot 20 μL of the bead-bound captured DNA as template into each of the two PCR tubes or wells. Add 20 μL of PCR-grade water to the negative control.

(e) Mix well by gently pipetting up and down five times.

(f) Store the remaining bead-bound captured DNA at −15 to −25 °C.

3. Performing the PCR amplification.

(a) Place the PCR tubes (or 96-well PCR plate) in a thermal cycler.

(b) Amplify the captured DNA using the following Post-Capture LM-PCR program: 45 s at 98 °C, followed by 16 cycles of 45 s at 98 °C, 30 s at 60 °C and 30 s at 72 °C, followed by 1 min at 72 °C, hold at 4 °C.

(c) Store the reaction at +2 to +8 °C (for up to 72 h) until ready for clean-up.

4. Purifying the amplified, captured bisulfite-converted sample using DNA purification beads (*see* **Note 9**).

(a) Allow the DNA Purification Beads to warm to room temperature for at least 30 min prior to use.

(b) Pool the like-amplified, captured bisulfite-converted DNA samples into a 1.5 mL tube (approximately 100 μL). Process the negative control in exactly the same way as the amplified, captured sample.

(c) Vortex the beads for 10 s before use to ensure a homogenous mixture of beads.

(d) Add 180 μL (1.8 × volume) DNA Purification Beads to the 100 μL pooled amplified, captured bisulfite-converted DNA samples.

(e) Vortex briefly and incubate at room temperature for 15 min to allow the DNA to bind to the beads.

(f) Place the tube containing the bead bound DNA in a magnetic particle collector.

(g) Allow the solution to clear.

(h) Once clear, remove and discard the supernatant being careful not to disturb the beads.

(i) Add 200 μL of freshly prepared 80% ethanol to the tube containing the beads plus DNA. The tube should be left in the magnetic particle collector during this step.

(j) Incubate at room temperature for 30 s.

(k) Remove and discard the 80% ethanol and repeat **steps i–j** for a total of two washes with 80% ethanol.

(l) Following the second wash, remove and discard all of the 80% ethanol.

(m) Allow the beads to dry at room temperature with the tube lid open for 15 min (or until dry) (*see* **Note 3**).

(n) Remove the tube from the magnetic particle collector and resuspend the DNA using 52 μL of PCR-grade water.

(o) Pipette up and down ten times to mix to ensure that all of the beads are resuspended.

(p) Incubate at room temperature for 2 min.

(q) Place the tube back in the magnetic particle collector and allow the solution to clear.

(r) Remove 50 μL of the supernatant that now contains the amplified, bisulfite-converted sample library and aliquot into a new 1.5 mL tube.

(s) Store the purified DNA sample at −15 to −25 °C.

5. Checking the quality of the amplified, captured bisulfite-converted DNA sample.

(a) Analyze 1 μL of the amplified, captured bisulfite-converted DNA sample and negative control using a DNA 1000 chip (Fig. 4) and measure the A_{260}/A_{280} ratio using a NanoDrop spectrophotometer to quantify the concentration of DNA and to determine the DNA quality. The negative control should not show significant amplification, which could be indicative of contamination. Amplified, captured bisulfite-converted DNA should exhibit the following characteristics:

- The A_{260}/A_{280} ratio should be 1.7–2.0.
- The LM-PCR yield should be ≥500 ng.
- The average DNA fragment length should be between 150 and 500 bp.

(b) The amplified, captured bisulfite-converted DNA meets the requirements, proceed to sequencing. If the amplified, captured bisulfite-converted DNA does not meet the A_{260}/A_{280} ratio requirement, purify again using the DNA Purification Beads (or alternatively, a second Qiagen QIAquick PCR Purification column).

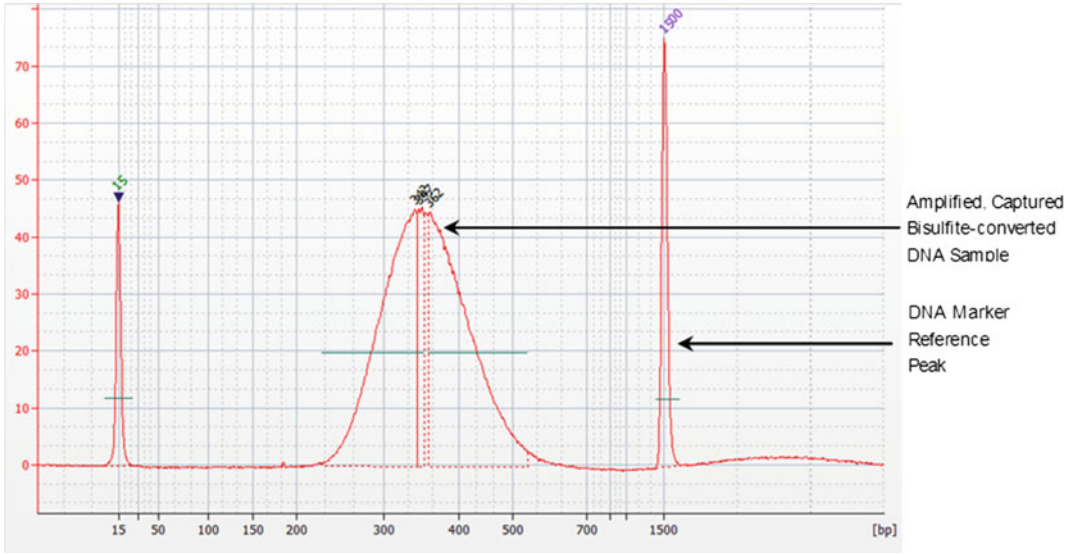

Fig. 4 Post-Capture LM-PCR. Example of an amplified, captured bisulfite-converted DNA sample analyzed using an Agilent DNA 1000 chip

6. Sequence the amplified, captured bisulfite-converted DNA sample (*see* **Note 10**).

3.7 Bisulfite Conversion and Capture Control (See Note 11)

1. Sequence reads should be aligned against the reference genome of the sample as well as against the lambda genome (NC_001416.1) (*see also* Chapter 5).
2. Once reads have been mapped, process the mapped reads using a methylation analysis package. The BSMAP alignment application (https://code.google.com/p/bsmap/) contains the methratio.py script, which determines methylation calls. A sample usage is:
   python2.6 methratio.py -d hg19_plus_NC001416.fa -s /usr/local/samtools -m 1 -z -i skip -o Sample.methylation_results.txt Sample.bam.
   This will produce a file with methylation calls for all C positions covered by mapped reads. Filter the result file to only include the methylation calls for NC_001416 between positions 4500 and 6500 (the region targets for capture).
   The first eight columns of the resulting file should look like the following:

Chr	Pos	Strand	Context	Ratio	Eff_CT_count	C_count	CT_count
NC_001416	4500	–	GCGGG	0.005	388	2	388
NC_001416	4501	–	CGGGT	0	392	0	392
NC_001416	4502	–	GGGTT	0	399	0	399
NC_001416	4512	–	TTGTG	0.007	437	3	437
NC_001416	4514	–	GTGCG	0.002	449	1	449
NC_001416	4515	+	TGCGC	0.007	538	4	538
NC_001416	4517	+	CGCTT	0	555	0	555
NC_001416	4521	+	TGCAG	0.003	575	2	575
NC_001416	4523	–	CAGGC	0.002	490	1	490
NC_001416	4524	–	AGGCC	0	497	0	497

Use the C_count and CT_count columns to calculate the percent conversion:

conversion rate = 1−(sum(C_count)/sum(CT_count).

For the above example, the conversion rate = 1−(13/4720) = 0.9972 = 99.72%.

3. Bisulfite conversion rates should generally be above 99.5% to be considered successful.

4 Notes

1. For the hybridization set up, 1 μg of amplified, bisulfite-converted sample library is recommended. Therefore, depending on the quality and quantity of the gDNA used during sample library construction and the results of the bisulfite conversion step, it may be necessary to increase the total number of PCR cycles to more than 12 cycles. Increasing the PCR cycles could result in an increase in the PCR duplicate rate (observed following sequencing). Additionally, it is possible to decrease the total number of PCR cycles if more than 1 μg of amplified, bisulfite-converted sample library is routinely obtained following the Pre-Capture LM-PCR.
2. Alternatively, samples can be purified using the Qiagen QIAquick PCR Purification Kit. If this purification method is chosen, follow the protocol detailed in the Qiagen QIAquick PCR Purification Kit guide with the following exception: Elute the amplified, bisulfite-converted sample library using PCR-grade water instead of Qiagen buffer EB.

3. Over drying of the beads can result in yield loss.
4. It is critical that the amplified, bisulfite-converted sample library is eluted with PCR-grade water and not Qiagen buffer EB or TE.
5. To prevent damage to the Hybridization Enhancing (HE) Oligos due to multiple freeze/thaw cycles, once resuspended the oligos can be aliquoted into smaller volumes to minimize the number of freeze/thaw cycles.
6. Denaturation of the DNA with high heat is not problematic after linker ligation because the hybridization utilizes single-stranded DNA.
7. These two reagents can be found in the SeqCap Hybridization and Wash Kit.
8. There is no need to elute the DNA off the beads. The beads plus captured DNA will be used as template in LM-PCR, as detailed below.
9. Alternatively, samples can be purified using the Qiagen QIAquick PCR Purification Kit. If this purification method is chosen, follow the protocol detailed in the Qiagen QIAquick PCR Purification Kit guide.
10. When working with bisulfite-converted sample libraries, some considerations need to be taken into account for sequencing when using an Illumina sequencing instrument. Due to the decreased nucleotide complexity in bisulfite-treated DNA, it may be necessary to perform one of the following options:
    (a) Designate a "control" lane when using a HiSeq2000 sequencing instrument. This lane should contain a DNA sample with a complex mixture of all four dinucleotides. Do not put your bisulfite-converted DNA sample in the control lane.
    (b) Mix in a sample with a more diverse representation of all four dinucleotides (with a different barcode index) into the bisulfite-converted DNA being sequenced. The diverse sample should be greater than or equal to 10% of the entire sample mixture.
    (c) In addition, due to the lower diversity, the input amount should be lowered slightly to generate a lower cluster count than standard. This will increase sequencing quality and result in more useful sequencing data.

    For best results, contact Illumina Technical Support prior to sequencing bisulfite-converted DNA.
11. This *Note* describes a procedure for using the Bisulfite conversion and Capture Control (BCC) that is provided in the SeqCap Epi Accessory Kit. This control consists of genomic DNA

from the Enterobacteria phage lambda, which is not naturally methylated at CpG dinucleotides in the *E. coli* host organism (*see also* Chapter 5). Bisulfite conversion of the phage lambda genomic DNA should thus result in all cytosines (C), including those in a CpG context, being converted to thymidines (T) after PCR amplification and sequencing. The completeness of C to T conversion in lambda DNA is used as a proxy for the completeness of bisulfite conversion of the experimental sample DNA in the mixture. If conversion of C to T in the lambda DNA is nearly complete, then observation of a CpG in the sample DNA is more likely to reflect true methylation status (i.e., true positives) rather than bisulfite conversion inefficiency (i.e., false positives). Although completeness of bisulfite conversion of non-CpG cytosines in the sample DNA itself (e.g., mitochondrial DNA) can also be used to estimate the completeness of bisulfite conversion using a reference sequence as a baseline, the use of a spike-in control will avoid variability related to sample quality and sequence polymorphism. Probes targeting a two-kilobase region between coordinates 4500 and 6500 bp of the lambda genome (NC_001416.1) are included by default in every SeqCap Epi design to specifically capture this region from the BCC. This streamlines the process of calculating bisulfite conversion by focusing analysis on a small and well-defined target.

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