Whole-genome Bisulfite Sequencing for Methylation Analysis

Preparing Samples for the Illumina Sequencing Platform

Introduction

This protocol explains how to prepare libraries of genomic DNA (gDNA) for whole-genome bisulfite sequencing (WGBS), a sequencing-based methylation analysis application. The libraries are prepared for subsequent cluster generation starting from sample DNA through adaptor ligation, library purification, and quantification.

Input gDNA (5 µg) is fragmented by Covaris shearing. The fragments are blunt-ended and phosphorylated, and a single 'A' nucleotide is added to the 3' ends of the fragments in preparation for ligation to a methylated adapter that has a single-base 'T' overhang. The ligation products are purified and size-selected by agarose gel electrophoresis. Size-selected DNA is bisulfite-treated and purified. The treated DNA is PCR-amplified to enrich for fragments that have adapters on both ends. The final purified product is then quantitated prior to cluster generation.

NOTE
It is essential to run a control lane on the same flowcell as the WGBS sample libraries. Because bisulfite treatment greatly decreases the C base frequency in the genomic sequence, the resulting libraries have an unbalanced base composition and are not optimal for base calls. Any genomic library containing balanced A,G,T, and C components (e.g., genomic resequencing, RNA-seq, ChIP-seq) can serve as a control lane for WGBS sample libraries.

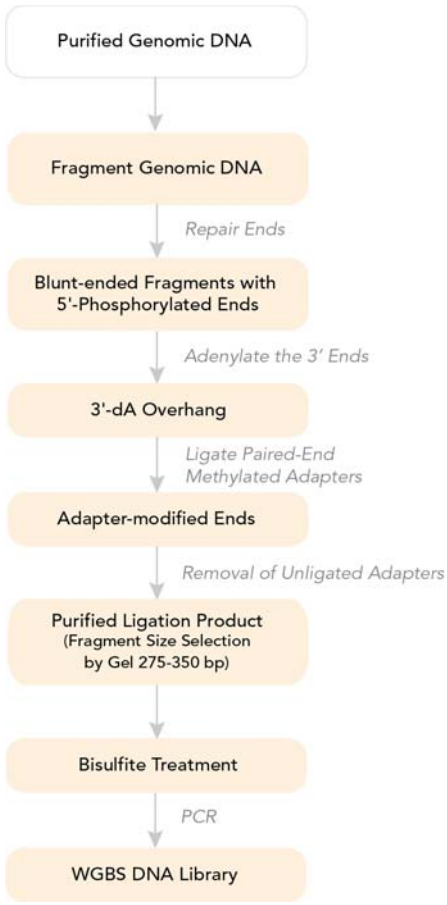
NOTE
The final concentration of the WGBS library is lower than 10 nM, therefore the subsequent cluster generation protocol must be modified as follows:

1. Add 1 µl 0.2N NaOH to 1 µl of the original WGBS library.
2. Let the tube stand for 5 minutes at room temperature.
3. Add 120 µl Hybridization buffer to the tube to dilute the library.
4. Place the tube on ice until till it is ready for cluster generation.

Sample Prep Workflow

The following figure illustrates the steps in the WGBS for Methylation protocol.

Figure 1 WGBS for Methylation Workflow



Best Practices

When preparing genomic DNA libraries for sequencing, you should always adhere to good molecular biology practices.

Liquid Handling

Good liquid handling measures are essential, particularly when quantifying libraries or diluting concentrated libraries for making clusters.

- Small differences in volumes (±0.5 µl) can sometimes give rise to very large differences in cluster numbers (~100,000).
- Small volume pipetting can be a source of potential error in protocols that require generation of standard curves, such as PicoGreen assays or qPCR, or those that require small but precise volumes, such as the Agilent BioAnalyzer.
- If small volumes are unavoidable, then due diligence should be taken to make sure that pipettes are correctly calibrated.
- Make sure that pipettes are not used at the volume extremes of their performance specifications.
- Care should be taken with solutions of high molecular weight double-stranded DNA (ds DNA). These can be viscous and not evenly dispersed, resulting in aliquot measurements that are not representative of the true concentration of the solution.
- To minimize pipetting errors, especially with small volume enzyme additions, prepare the reagents for multiple samples simultaneously. As a result, you pipette once from the reagent tubes with a larger volume, rather than many times with 1 µl volumes. Prepare a master mix of enzymes, water, buffer, etc. and aliquot this in a single pipetting movement to individual samples to standardize across multiple samples.

Potential DNA Contaminants

Incorrect DNA quantification may result from DNA contamination.

- Open only one adapter at the time.
- Pipette carefully to avoid spillage.
- Clean pipettes and change gloves between handling different adapter stocks.
- Clean work surfaces thoroughly before and after the procedure.
- Change gloves between handling different adapter stocks.
- Contamination may be result from interference from superfluous nucleic acids in a sample (e.g., RNA, small nucleic acid fragments, nucleotides, single-stranded DNA), excess proteins, or other contaminating materials.
- DNA quality may also affect the quantity of usable DNA in a sample. For example, if the DNA is damaged (e.g., heavily nicked or containing extensive apurinic/apyrimidinic sites), then many of these fragments may fail during library preparation.
- High molecular weight dsDNA derived from host genomes can also interfere with accurate quantification. For example, bacterial artificial chromosomes (BACs) and other bacterially-derived plasmids usually contain a few percent of the chromosomal DNA from the host cells, despite the best purification efforts. These sequences may ultimately give rise to unwanted clusters on a flow cell lane. However, this contamination can be accurately quantified by analyzing aligned reads generated during sequencing against known bacterial sequences and subtracting these out.

- High molecular weight contamination may also be estimated prior to library preparation using qPCR assays designed to target unique chromosomal markers.

Temperature Considerations

Temperature is an important consideration for making DNA libraries:

- Keep libraries at temperatures ≤37°C.
- Avoid elevated temperatures, particularly in the steps preceding the adapter ligation.
- DNA fragments that have a high AT content are more likely to denature into single strands than GC-rich fragments, which can result in an increased probability of creating a bias in the sequencing coverage.
- Take care not to denature the library prior to the agarose gel electrophoresis process.

DNA Input Recommendations

Input DNA Quantification

The ultimate success or failure of a library preparation strongly depends on using an accurately quantified amount of input DNA, particularly when starting quantities are <1 µg. Thus, correct quantification of gDNA is essential. This protocol is optimized for 5 µg input DNA.

Assessing DNA Quantity and Quality

Absorbance measurements at 260 nm are commonly used to quantify DNA. The ratio of absorbance at 260 nm to absorbance at 280 nm is used as an indication of sample purity, and values of 1.8–2.0 are considered indicative of relatively pure DNA. However, both measurements can be compromised by the presence of RNA or small nucleic acid fragments such as nucleotides. Thus, gDNA samples should be carefully collected to ensure that they are free of contaminants, and the most accurate spectrophotometric method available should be used to quantify the input gDNA.

DNA quantification methods that rely on intercalating fluorescent dyes measure only double-stranded DNA and are less subject to excess nucleic acids. However, these methods require the preparation of calibration curves and are highly sensitive to pipetting error. Ensure that pipettes are correctly calibrated and are not used at the volume extremes of their performance specifications.

Gel electrophoresis is a powerful means for revealing the condition (even the presence or absence) of DNA in a sample. Impurities, such as detergents or proteins, can be revealed by smearing of DNA bands. RNA, which interferes with 260 nm readings, is often visible at the bottom of a gel. A ladder or smear below a band of interest may indicate nicking or other damage to DNA. Where possible, or necessary, a gel should be run to assess the condition of the DNA sample.

Consistency of Results

Given that fluorescent methods only measure double stranded DNA and 260 nm determinations measure both single and double stranded DNA, fluorescence analysis can be performed to assess the sample denaturation state (i.e. single or double stranded). Sample preparation can begin with a fixed amount of double-stranded DNA prior to fragmentation and cleanup, and thereafter, can be measured by 260 nm determination. If the DNA amount is less than expected, the amount of input DNA can be adjusted before proceeding to the end-repair steps. A further validation step can be performed by analyzing an aliquot for the presence of contaminants by electrophoresis or using an automated instrument, such as the Agilent Bioanalyzer.

Consumables and Equipment

Check to make sure that you have all of the necessary user-supplied consumables and equipment before proceeding to sample preparation.

Table 1 User-Supplied Consumables

Consumable	Supplier
0.2 ml nuclease-free thin-wall PCR tubes	USA Scientific, catalog # 1402-4700
2.0 ml DNA LoBind tubes	Eppendorf, catalog # 022431048
50 bp Redi-Load DNA ladder	NEB, catalog # N3231L
50X TAE Buffer	BIO-RAD, catalog # 161-0743
100 bp Redi-Load DNA ladder	NEB, catalog # N3236L
Certified low range ultra agarose	BIO-RAD, catalog # 161-3106
Clean scalpels	General lab supplier
Distilled water	General lab supplier
DNeasy Kit (or equivalent)	QIAGEN, catalog # (choose one): 69581 (4 samples) 69582 (12 samples) 69504 (50 samples) 69506 (250 samples)
EpiTect Bisulfite Kit	QIAGEN, catalog # 59104
Ethanol	General lab supplier
GelPilot DNA loading Dye, 5x	QIAGEN, catalog # 239901
MicroTube (6x16mm), AFA fiber with crimp-cap	Covaris, part # 520052
MinElute PCR Purification Kit	QIAGEN, catalog # 28004
Paired-End Sample Prep Kit	Illumina, catalog # PE-102-1001 (10 samples) or PE-102-1002 (40 samples)
PfuTurbo Cx Hotstart DNA Polymerase	Stratagene, catalog # 600410
Purified DNA (5 µg,) DNA should be as intact as possible, with an OD260/280 ratio of 1.8–2.0	User supplied
QIAquick Gel Extraction Kit	QIAGEN, catalog # 28704
QIAquick PCR Purification Kit	QIAGEN, catalog # 28104
SYBR Green	Invitrogen, catalog # S7563
TruSeq DNA Sample Prep Kit v2	Illumina, catalog # FC-121-2001 (Set A) or FC-121-2002 (Set B)
Ultra Pure Water	General lab supplier
Unmethylated lambda gDNA	Promega, catalog # D1521

Table 2 User-Supplied Equipment

Equipment	Supplier
[Optional] 2100 Bioanalyzer Desktop System	Agilent, part # G2940CA
[Optional] Agilent DNA 1000 Kit	Agilent, part # 5067-1504
[Optional] Agilent High Sensitivity DNA Kit	Agilent, part # 5067-4626
Benchtop microcentrifuge	General lab supplier
Benchtop centrifuge with swing-out rotor (e.g., Sorvall Legend RT)	General lab supplier
One of the following Covaris systems: S2 S220 E210 E220	 Covaris, part # S2 Covaris, part # S220 Covaris, part # E210 Covaris, part # E220
Dark Reader transilluminator or a UV transilluminator	Clare Chemical Research, catalog # D195M
Electrophoresis unit	General lab supplier
Gel trays and tank	General lab supplier
Thermal cycler (with heated lid)	General lab supplier

Fragment DNA

This process describes how to optimally fragment the gDNA to 250 bp (peak size). Covaris shearing generates dsDNA fragments with 3' or 5' overhangs.

Consumables

- 0.2 ml nuclease-free thin-wall PCR tube
- Covaris Tubes
- DNeasy Kit (or equivalent)
- gDNA
- Ultra Pure Water
- Unmethylated Lambda gDNA

NOTE
Unmethylated lambda gDNA is used as the bisulfite conversion rate control and the data analysis pipeline for this protocol was designed with this control.

Procedure

1. Prepare 5 µg of gDNA using a QIAGEN DNeasy or equivalent kit.
2. Normalize the gDNA samples and 0.5% W/W lambda DNA with QIAGEN EB to a final volume of 50 µl.
3. Fragment the DNA using the following settings:

Function	S2/E210 Settings	Function	S220/E220 Settings
DNA volume	50 µl	DNA volume	50 µl
Duty cycle	20%	Duty cycle	20%
Intensity	4.0	Peak Incident Power	140
Cycles per burst	200	Cycles per burst	200
Duration	60 seconds	Duration	60 seconds
Mode	Frequency sweeping	Mode	Frequency sweeping
Power	36W	Temperature	5.5° to 6°C
Temperature	5.5° to 6°C		

4. Verify the size distribution of the library by running a 1 µl aliquot on a Agilent Technologies 2100 Bioanalyzer using a Agilent DNA 1000 chip.
5. Transfer 49 µl of the fragmented DNA to a 0.2 ml nuclease-free thin-wall PCR tube.

Perform End Repair

This procedure converts the overhangs resulting from fragmentation into blunt ends using T4 DNA polymerase and Klenow enzyme. The 3' to 5' exonuclease activity of this mix removes the 3' overhangs and the polymerase activity fills in the 5' overhangs.

Consumables

- Paired-End Sample Prep Kit content:
  - 10 mM dNTP Mix
  - Klenow Enzyme
  - T4 DNA Ligase Buffer with 10 mM ATP
  - T4 DNA Polymerase
  - T4 PNK
  - Ultra-pure Water
- QIAquick PCR Purification Kit

Procedure

1 Prepare the reaction mix on ice in the following order:

Reagent	Volume (µl)
DNA Sample	49
Water	26
T4 DNA Ligase Buffer with 10 mM ATP	10
10 mM dNTP Mix	4
T4 DNA Polymerase	5
Klenow Enzyme	1
T4 PNK	5
Total Volume	**100**

2 Vortex the reaction mix briefly to mix thoroughly.

3 Incubate on the thermal cycler for 30 minutes at 20°C.

4 Follow the instructions in the QIAquick PCR Purification Kit to purify on one QIAquick column, eluting in 32 µl of QIAGEN EB.

SAFE STOPPING POINT
If you do not plan to proceed to *Adenylate 3' Ends* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer. When proceeding, thaw the samples on ice.

Adenylate 3' Ends

A single ‘A’ nucleotide is added to the 3’ ends of the blunt fragments to prevent them from ligating to one another during the adapter ligation reaction. A corresponding single ‘T’ nucleotide on the 3’ end of the adapter provides a complementary overhang for ligating the methylated adapter to the fragment. This strategy ensures a low rate of chimera (concatenated template) formation.

Consumables

- MinElute PCR Purification Kit
- Paired-End Sample Prep Kit content:
  - Klenow Buffer
  - 1 mM dATP
  - Klenow Exo -

NOTE

This process requires a QIAquick MinElute column rather than a normal QIAquick column.

Procedure

1. Prepare the reaction mix on ice in the following order:

Reagent	Volume (µl)
DNA Sample	32
Klenow Buffer	5
1 mM dATP	10
Klenow Exo -	3
Total Volume	**50**

2. Incubate on the thermal cycler for 30 minutes at 37°C.
3. Follow the instructions in the MinElute PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 10 µl of QIAGEN EB.

Ligate Adapters

This process ligates adapters to the ends of the DNA fragments. The reaction adds distinct sequences to the 5′ and 3′ ends of each strand in the genomic fragment.

Consumables

- Paired-End Sample Prep Kit content:
  - DNA Ligase Buffer, 2X
  - DNA Ligase
- TruSeq DNA Sample Prep Kit v2 content:
  - DNA Adapter Index (AD001–AD016, AD018–AD023, AD025, or AD027)
- MinElute PCR Purification Kit

NOTE
Any DNA Adapter Index can be used, however indexes 2 or 5 are recommended for this protocol.

Procedure

A molar excess of adapter to fragments is used to increase the yield of adapter ligation to both ends of the DNA fragments. If the ratio is too high, then the yield of adapter dimers also increases. The volume of adapter oligo mix added in the following procedure is recommended for an initial input DNA quantity of 5 µg.

1 Prepare the reaction mix on ice in the following order:

Reagent	Volume (µl)
DNA Sample	10
DNA Ligase Buffer 2X	25
DNA Adapter Index	10
DNA Ligase	5
Total Volume	**50**

2 Incubate on the thermal cycler for 30 minutes at 20°C.

3 Follow the instructions in the MinElute PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 10 µl of QIAGEN EB.

SAFE STOPPING POINT
If you do not plan to proceed to *Purify Ligation Products* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer. When proceeding, thaw the samples on ice.

Purify Ligation Products

This process purifies the products of the ligation reaction on a gel and removes unligated adapters, removes any adapters that may have ligated to one another, and selects a 275–350 bp size-range of DNA fragments for bisulfite treatment.

NOTE

Test your electrophoresis unit in advance to make sure that you can readily resolve DNA in the range below 1000 base pairs. The DNA smear should be sufficiently resolved to enable you to excise a narrow band of a chosen size with a standard deviation as low as 5% of the median (i.e., a gel slice at 400 bp, where +/- one standard deviation is equivalent to a size range of 380–420 bp). The conditions described are typical and validated gel electrophoresis conditions.

Perform gel electrophoresis and band excision after adapter ligation to remove excess adapter and adapter dimers and to tighten the range of fragment sizes. Ligation reaction products are separated on an agarose gel and a ~2 mm wide gel slice containing DNA of the desired size is excised.

NOTE

These procedures have only been verified using the user-supplied consumables specified in this guide and by performing the gel-method specified below. Any deviation from these materials and procedures may result in incorrect size excision or require additional user optimization.

Consumables

- 2.0 ml DNA LoBind Tubes (2)
- Certified Low Range Ultra Agarose
- Clean Scalpels
- 50x TAE Buffer
- Distilled Water
- GelPilot DNA Loading Dye, 5x
- 100 bp Redi-Load DNA Ladder
- 50 bp Redi-Load DNA Ladder
- SYBR Green
- QIAquick Gel Extraction Kit

Procedure

1. Prepare a 100 ml, 2% agarose gel with distilled water and TAE according to the manufacturer's instructions. The final concentration of TAE should be 1X.
2. Make a 1:100 dilution of 10,000X SYBR Green using QIAGEN EB.
3. Cast the gel using a comb that can accommodate 30 µl in each well.
   Recommended well size: 1 mm (length) x 8 mm (width) x 7 mm (height).
4. Dilute the 50 bp Redi-Load Ladder to 100 ng/ µl with GelPilot DNA Loading Dye.
5. Dilute the 100 bp Redi-Load Ladder to 100 ng/ µl with GelPilot DNA Loading Dye.
6. Add 2 µl of the SYBR Green dilution to 10 µl of the 50 bp Redi-Load Ladder.

7 Add 2 µl of the SYBR Green dilution to 10 µl of the 100 bp Redi-Load Ladder.

8 Load 12 µl of each ladder onto one lane of the gel, flanking the library on both sides.

NOTE

Flanking the library on both sides with ladders may make the library excision easier.

9 Add 6 µl of GelPilot DNA Loading Dye to 10 µl of the DNA from the purified ligation reaction. Gently pipette the entire volume up and down 10 times to mix thoroughly.

10 Add 3 µl of the SYBR Green dilution to the 16 µl of DNA and GelPilot DNA Loading Dye mixture. Gently pipette the entire volume up and down 10 times to mix thoroughly.

11 Load the entire sample (19 µl) onto the middle lane of the gel.

12 Run gel at 120 V for 10 minutes, then 60 V for 120 minutes (6 V/cm) or until the orange dye reaches to the bottom of the gel.

13 View the gel on a Dark Reader transilluminator or a UV transilluminator.

14 Photograph the gel before a slice is excised.

15 Excise a gel slice of the sample lane at exactly 275–350 bp using a clean scalpel. Use the DNA ladder as a guide.

16 Place the gel slice in a new 2.0 ml DNA LoBind tube.

17 Discard the scalpel to avoid sample cross-contamination.

18 Photograph the gel after the slice was excised.

19 Follow the instructions in the QIAquick Gel Extraction Kit to purify on one QIAquick column.Weigh the gel slice as indicated in the instructions, in order to calculate how much initial buffer to add, eluting in 20 µl of QIAGEN EB twice and combine them into one tube.

The final volume should be 40 µl for each band region.

SAFE STOPPING POINT

If you do not plan to proceed to *Bisulfite Treat Ligation Products* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer. When proceeding, thaw the samples on ice.

Bisulfite Treat Ligation Products

In this procedure, the size selected, methyl-adapter ligated gDNA fragments are treated with bisulfite to convert any un-methylated Cytosine to Thymidine. The procedures were adapted from the EpiTect Bisulfite Kit and modified.

Consumables

- Ethanol
- EpiTect Bisulfite Kit
- MinElute PCR Purification Kit

Procedure

1. Follow the instructions in the EpiTect Bisulfite Kit to prepare buffer BW, buffer BD, and carrier RNA.
2. Follow the instructions in the EpiTect Bisulfite Kit to prepare buffer BL and bisulfite mix on the date of experiment.
3. Follow the instructions in the EpiTect Bisulfite Kit, to prepare the reaction mix on ice:

Reagent	Volume (µl)
DNA Sample	35–40
RNase Free Water	0–5
Bisulfite Mix	85
DNA Protection Buffer	15
Total Volume	**140**

4 Incubate the reaction mix tube in the pre-programmed thermal cycler as follows, with the lid heated and closed. The complete cycle takes approximately 14 1/2 hours:

	Procedure	Temperature	Time
	Denaturation	95°C	5 minutes
	Incubation	60°C	25 minutes
	Denaturation	95°C	5 minutes
	Incubation	60°C	85 minutes
	Denaturation	95°C	5 minutes
	Incubation	60°C	175 minutes
X 3	Denaturation	95°C	5 minutes
X 3	Incubation	60°C	180 minutes
	Hold	20°C	Indefinite

NOTE
Do not hold for more than five hours.

5 Follow the instructions in the EpiTect Bisulfite Kit Handbook (09/2009) to purify the bisulfite converted DNA (use the purification protocol for bisulfite conversion of DNA isolated from FFPE Tissue Samples), replacing step 12 as follows:

Add 500 buffer BD (desulfonation buffer) to the spin column, and incubate for 20 minutes at room temperature.

6 Elute in 20 µl of QIAGEN EB twice and combine them into one tube.

The final volume should be 40 µl for each band region.

NOTE
It is important to purify the eluted DNA from the EpiTect Bisulfite Kit with a MinElute column. Otherwise, the yield of the subsequent PCR amplification will be compromised.

7 Further purify and concentrate using a MinElute column, following the instructions in the MinElute PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 15 µl of QIAGEN EB.

SAFE STOPPING POINT
If you do not plan to proceed to *Enrich DNA Fragments* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or for up to four weeks (Bisulfite treated DNA is not suitable for storage periods longer than four weeks.) When proceeding, thaw the samples on ice.

Enrich DNA Fragments

This process uses PCR to selectively enrich those DNA fragments that have methylated adapter molecules on both ends and to amplify the amount of DNA in the library for accurate quantification. The PCR is performed with two primers that anneal to the ends of the adapters.

Consumables

- MinElute PCR Purification Kit
- Paired-End Sample Prep Kit content:
  - 10 mM dNTP Mix
- PfuTurbo Cx Hotstart DNA Polymerase Kit content:
  - PfuTurbo Cx Hotstart DNA Polymerase
  - Pfu Turbo Cx Reaction Buffer
- TruSeq DNA Sample Prep Kit v2 content:
  - PCR Primer Cocktail (PPC)
- Ultra Pure Water

Procedure

The volume of methyl-adapter ligated fragments added to the PCR reaction below are based on an initial input DNA quantity of 5 µg. With 12–15 µl of bisulfite treatment elution from the *Bisulfite Treat Ligation Products* procedures, three independent PCR reactions will be set to maximize the library yield and diversity. The final library volume will be 3 x 15 µl in three different tubes.

CAUTION

To avoid sample cross-contamination, set up PCR reactions (all components except the template DNA) in a designated clean area, preferably a PCR hood with UV sterilization and positive air flow.

1 Prepare the reaction mix on ice in a 200 µl thin wall PCR tube in the following order:

Reagent	Volume (µl)
DNA	4
Ultra Pure Water	33.75
Pfu Turbo Cx Reaction Buffer	5
10 mM dNTP Mix	1.25
PCR Primer Cocktail	5
PfuTurbo Cx Hotstart DNA Polymerase	1
Total Volume	**50**

2 Vortex the reaction mix briefly to mix thoroughly.

3 Amplify using the following PCR process with a heated lid:

- **a** 5 minutes at 95°C
- **b** 30 seconds at 98°C
- **c** 4 cycles of:
  10 seconds at 98°C
  30 seconds at 65°C
  30 seconds at 72°C
- **d** 5 minutes at 72°C
- **e** Hold at 4°C

NOTE
The recommended number of PCR cycles is 4–8, but the number of cycles should be minimized to avoid skewing the representation of the library. Use eight cycles when the yield is low.

4 Follow the instructions in the MinElute PCR Purification Kit to purify on one QIAquick MinElute column, eluting in 15 µl of QIAGEN EB.

SAFE STOPPING POINT
If you do not plan to proceed to *Validate Library* immediately, the protocol can be safely stopped here. If you are stopping, store the samples at -15° to -25°C overnight or longer. When proceeding, thaw the samples on ice.

Validate Library

Perform the following procedures for quality control analysis on your sample library and quantification of the WGBS DNA library templates.

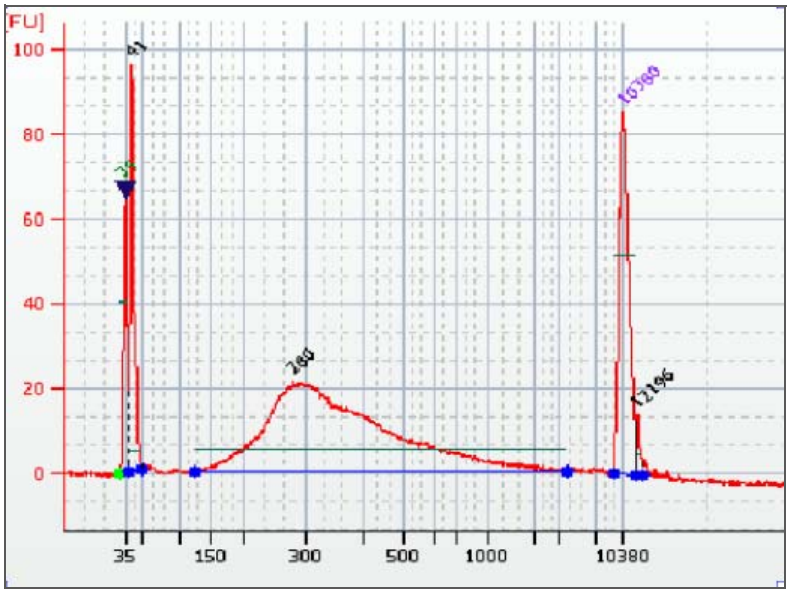
Quantify Libraries

In order to achieve the highest quality of data on Illumina sequencing platforms, it is important to create optimum cluster densities across every lane of every flow cell. This requires accurate quantification of DNA library templates. As described for sample input quantification, any method of measuring DNA concentration has certain advantages and potential drawbacks. Due to low yield, to quantify the WGBS library run an aliquot of the DNA library on a Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip.

Quality Control

To quantify and verify the size of your PCR enriched fragments, check the concentration and template size distribution by running an aliquot of the DNA library on a Agilent Technologies 2100 Bioanalyzer using a High Sensitivity DNA chip. Load 1 µl of the library on the Agilent High Sensitivity DNA chip.

Figure 1 Example of DNA Library Distribution for WGBS for Methylation



qPCR

Quantitative real-time PCR (qPCR) is an alternative method of quantifying DNA that measures the relationship between the initial concentration of a template and how its concentration changes during progressing cycles of thermal amplification. qPCR quantification is not recommended for the WGBS for Methylation protocol due to complications in the library components.

Cluster Generation

It is essential to run a control lane on the same flowcell as the WGBS for Methylation sample libraries. Because bisulfite treatment greatly decreases the C base in the genomic sequence, the resulting three based (T, A and G) libraries are not optimal for base calls. Any genomic library containing balanced A,G,T, and C components (e.g., genomic resequencing, RNA-seq, ChIP-seq) could be served as a control lane for WGBS for Methylation sample libraries.

NOTE
The final concentration of the WGBS library is lower than 10 nM, therefore the subsequent cluster generation protocol must be modified as follows:

1. Add 1 µl 0.2N NaOH to 1 µl of the original WGBS library.
2. Let the tube stand for 5 minutes at room temperature.
3. Add 120 µl Hybridization buffer to the tube to dilute the library.
4. Place the tube on ice until till it is ready for cluster generation.

DNA Template Storage

Store prepared DNA template at a concentration of 10 nM. Adjust the concentration for your prepared DNA samples (or pools of samples) to 10 nM using Tris-HCl 10 mM, pH 8.5. For long-term storage of DNA samples at a concentration of 10 nM, add Tween 20 to the sample to a final concentration of 0.1% Tween. This helps to prevent adsorption of the template to plastic tubes upon repeated freeze-thaw cycles, which would decrease the cluster numbers from a sample over time.