diagenode

Innovating Epigenetics Solutions

Premium WGBS Kit

Whole Genome Bisulfite Sequencing

Cat. No. C02030034 (8 rxns)

USER GUIDE

Version 2 12_2018

Please read this manual carefully
before starting your experiment

Contents

Introduction

Whole Genome Bisulfite Sequencing (WGBS) is a single nucleotide resolution technique that allows the user to study DNA methylation sites and their role in gene regulation. The Diagenode's **Premium WGBS Kit** is designed to prepare single and paired-end bisulfite converted DNA libraries for sequencing using Illumina® platforms. It has also been validated for the preparation of bisulfite converted library from ChIP'd samples in order to perform **ChIP-Bis-Sequencing**. For Reduced Representation Bisulfite Sequencing (RRBS) experiments use the Diagenode's Premium RRBS Kit (Cat. No.C02030032 and C02030033).

The Diagenode's Premium WGBS Kit contains specially designed enzymes and buffers needed for genome-wide bisulfite sequencing. This kit includes:

- a highly efficient adapter ligation step which results in superior library preparations and a maximum number of unique sequencing reads
- a specially designed WGBS enzymatic ligation mix which allows users to perform ligations with longer adapters and better ligation efficiencies
- the MethylTaq Plus 2X Master Mix, a robust polymerase designed to handle bisulfite converted DNA.

These optimizations permit to start a WGBS experiment with amounts of DNA as low as **5 ng**.

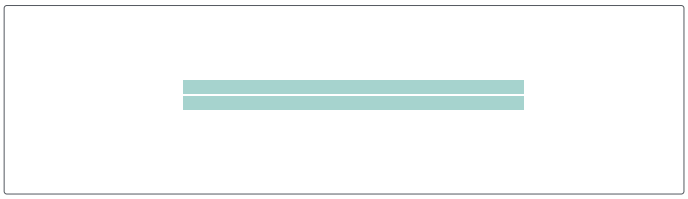
Moreover, the Premium WGBS Kit simplifies the workflow by using master mixed reagents and magnetic bead based cleanup, reducing pipetting and eliminating time consuming steps in library preparation.

Kit method overview & time table

Genomic DNA

Fragment

STEP 1



End-repair

1 hour (Optional Stop Point)

STEP 2

Adenylation

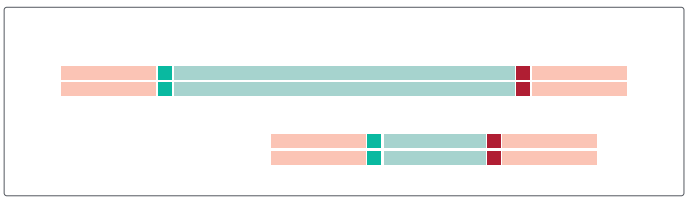
30 min

STEP 3

Ligation of methylated adapters

45 min (Optional Stop Point)

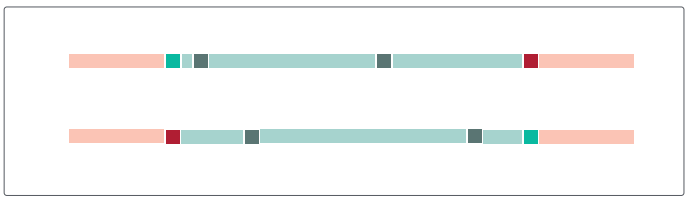
STEP 4



Gel-free size selection

30 min (Optional Stop Point)

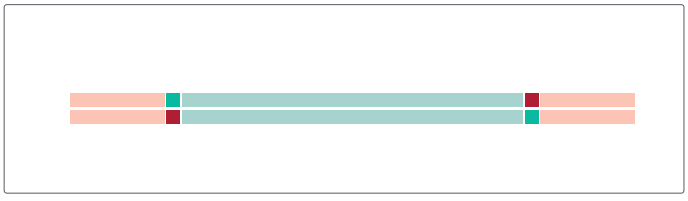
STEP 5



Bisulfite conversion

4 h

STEP 6



Amplification

1 h (Optional Stop Point)

Ready for sequencing

LEGEND

N T A U Adapters with Cluster Sequence

Kit materials

The Premium WGBS Kit contains enough material to prepare 8 genomic DNA samples for Illumina compatible sequencing. The shelf life of all reagents is 6 months when stored properly.

> ***NOTE**: The kit is provided with one adapter containing a barcode. In case of multiplexing, more barcodes are available separately (see optional supplies page 7).*

⚠ Store the components at the indicated temperature upon receipt.

Table 1. Components supplied with the Premium WGBS Kit

Description	Quantity	Storage
Water (white)	1.5 mL	-20°C
WGBS End Repair Buffer Mix (clear)	56 µL	-20°C
WGBS End Repair Enzyme Mix (clear)	24 µL	-20°C
WGBS Adenylation Mix (red)	36 µL	-20°C
WGBS Ligation Mix (purple)	252 µL	-20°C
WGBS Adapter 25 µM (purple)*	20 µL	-20°C
WGBS Adapter Dilution Buffer (purple)	500 µL	-20°C
Resuspension Buffer (white)	2 mL	-20°C
BS Conversion reagent	1 tube	Room temperature
BS Dilution Buffer (black)	300 µL	Room temperature
BS Solubilization Buffer (black)	790 µL	Room temperature
BS Reaction Buffer (black)	160 µL	Room temperature
BS Desulphonation Buffer	1600 µL	Room temperature
BS Elution Buffer (white)	176 µL	Room temperature
BS Spin Columns	8 columns	Room temperature
BS Collection Tubes	8 tubes	Room temperature
WGBS Primer Mix (green)	16 µL	-20°C
MethylTaq Plus 2X Master Mix (green)	200 µL	-20°C

** The provided adapter contains the barcode #24 with the following sequence: GGTAGC*

Required materials not provided

- Gloves to wear at all steps
- Ethanol 100% (room temperature)
- Nuclease-free water
- 8-tube strips or 0.2 mL PCR tubes
- Agencourt AMPure XP 5 mL (Beckman Coulter Genomics, Cat. No. A63880))
- DiaMag02 Magnetic Rack (Diagenode, Cat. No. B04000001)
- Heat block
- Thermocycler
- 2, 10, 20, 200 and 1000 µL pipettes
- Nuclease-free barrier pipette tips
- Microcentrifuge
- 1.5 mL nuclease-free microcentrifuge tubes

Equipment required for quality control:

- Fluorescence-based assay for DNA concentration measurement, e.g. the Qubit High Sensitivity assay (Life Technologies Cat. No. Q32851)
- Library analysis assay such as Agilent High Sensitivity DNA Kit for BioAnalyzer (Agilent, Cat. No. 5067-4626)

Optional supplies available separately :

- Bisulfite conversion reagent for RRBS (8 rxns) - Cat. No. C02030035
- For multiplexing: Premium WGBS Indexes
  - 6 indexes (48 rxns) - Cat. No. C05010032
  - 12 indexes (96 rxns) - Cat. No. C05010033
  - 24 indexes (192 rxns) - Cat. No. C05010034

Remarks before starting

Warnings and precautions

- DTT in buffers may precipitate after freezing. If precipitate is seen, vortex buffer for 1-2 minutes or until the precipitate is in solution. The performance of the buffer is not affected once precipitate is in solution.

- Do not heat the WGBS Adapters above room temperature.

- If starting with a DNA input amount greater than or less than 1 µg, adjust the WGBS Adapter volume to preserve the insert to adapter ratio.

Starting Material

The Premium WGBS Kit has been optimized using fragmented DNA.

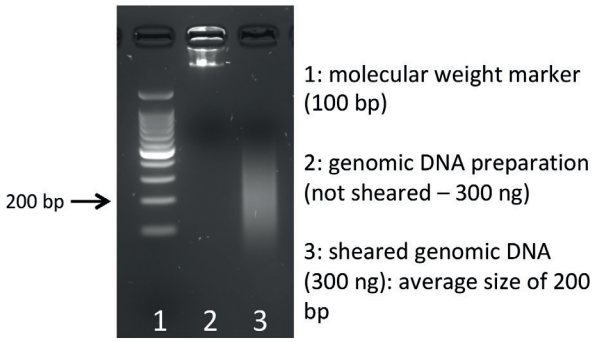
Genomic DNA must be randomly sheared by sonication to generate fragments around 200 bp (see example below). All the DNA shearing protocols using Diagenode's sonication devices are available on our website: www.diagenode.com.

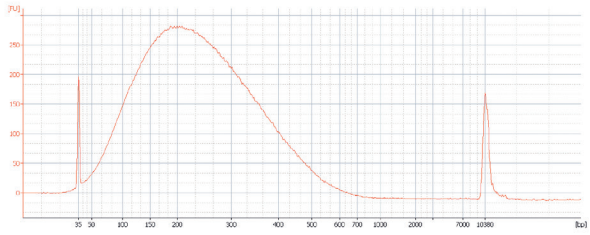
Example of shearing using the Bioruptor Pico for WGBS:

The genomic DNA was diluted in TE buffer to reach a concentration of 100 ng/µL and 100 µL were sheared in a 0.65 ml Bioruptor® Microtube (Cat. No. C30010011).

The following program was used:

- Cycles: [30 seconds "ON" & 30 seconds "OFF"]
- 13 cycles





Agilent High Sensitivity DNA chip profile of sheared genomic DNA: peak at 200 bp

Figure 1: DNA shearing using Bioruptor® Pico

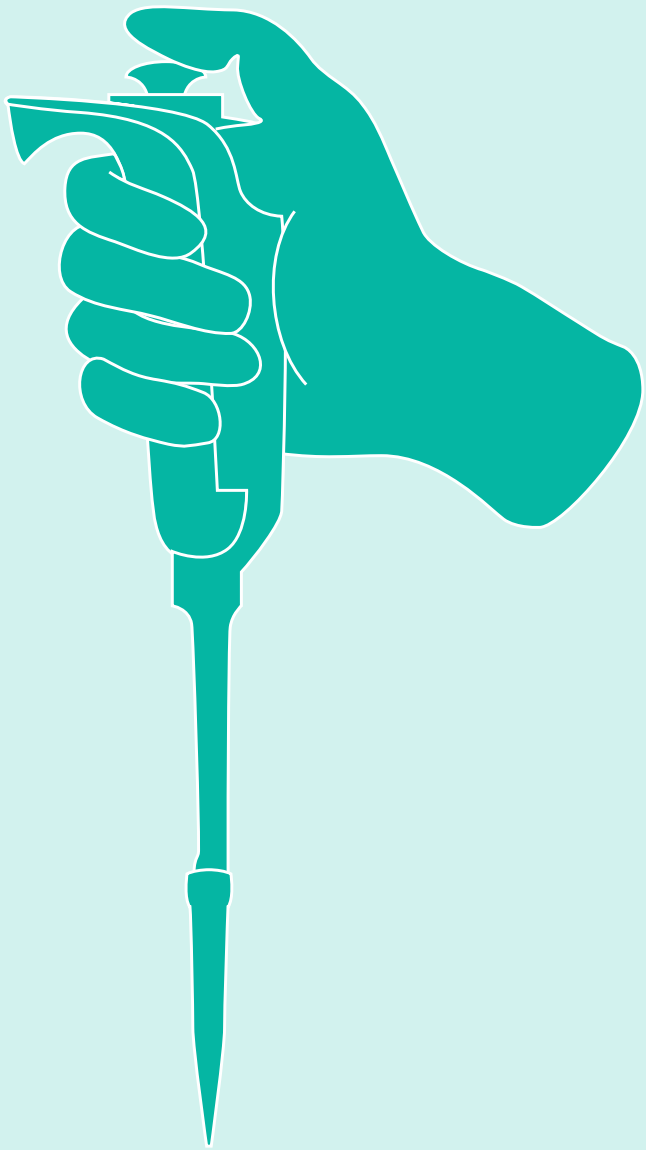
The Diagenode's Premium WGBS Kit has also been validated for ChIP-Bis-sequencing experiments. Thus the starting material can be some immunoprecipitated DNA after ChIP experiments against histone marks. For ChIP experiments we recommand the iDeal ChIP-seq kit for Histones (Cat. No. C01010051).

Starting inputs from 5 ng to 1 µg of fragmented genomic DNA or ChIP'd DNA are compatible with this kit.

Reagent Preparation

- Allow Agencourt AMPure XP Beads to come to room temperature and vortex the beads until liquid appears homogenous before every use.
- For best results, the BS Conversion Reagent should be used immediately after resolubilization. If all reactions are not performed the same day, it is possible to buy some more BS Conversion Reagent as a separate product: Bisulfite Conversion Reagent for RRBS (Cat. No. C02030035).

PROTOCOL



STEP 1

End repair

1 hour

1.1 For each sample, combine the following reagents on ice in a 8-tube strip or in a 0.2 ml.

	Per IP
Nuclease-free water (white cap)	_ µL
Fragmented or ChIP'd genomic DNA (5 ng - 1 µg)	_ µL
WGBS End Repair Buffer Mix (clear cap)	7 µL
WGBS End Repair Enzyme Mix (clear cap)	3 µl
TOTAL	**50 µL**

1.2 Mix thoroughly by pipetting.

1.3 Incubate on a thermocycler for 30 minutes at 22°C.

1.4 Add **90 µL of AMPure XP Beads** to each sample and mix thoroughly by pipetting.

1.5 Incubate at room temperature for 5 minutes.

1.6 Place the 8-tube strip on the DiaMag02 at room temperature for 5 minutes or until the supernatant appears clear.

1.7 Remove and discard clear supernatant. Take care not to disturb beads. Some liquid may remain in the tube.

1.8 Add **200 µL of freshly prepared 80% ethanol** to each magnetic bead pellet and incubate at room temperature 30 seconds. Carefully,

remove ethanol by pipette.

1.9 Repeat previous step, for a total of 2 ethanol washes. Ensure all ethanol has been removed.

1.10 Remove the tube from the DiaMag02 and let dry at room temperature for 3 minutes.

1.11 Resuspend dried beads with **17 µL of Resuspension Buffer**. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the tube.

1.12 Incubate resuspended beads at room temperature for 2 minutes.

1.13 Place tube on DiaMag02 at room temperature for 5 minutes or until the sample appears clear.

1.14 Transfer **16 µL of clear sample** to new 0.2 mL tube.

1.15 If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20°C. To restart, thaw frozen samples on ice before proceeding.

STEP 2

Adenylation

30 minutes

2.1 Combine the following reagents in a 0.2 mL tube or 8-tube strip:

	Per IP
End-Repaired DNA (from Step 2)	16 µL
WGBS Adenylation Mix (red cap)	4.5 µL
TOTAL	**20.5 µL**

2.2 Mix thoroughly by pipetting.

2.3 Incubate on a thermocycler for 30 minutes at 37°C.

STEP 3

Adapter Ligation

 *45 minutes*

If starting with less than 1 µg of input DNA, follow the 'Table 2 below to dilute the methylated adapters. This is important for retaining the optimal input:adapter ratio.

Table 2. Adapter dilution

Input DNA	10 ng	100 ng	250 ng	500 ng
WGBS Adapter	1 µL	1 µL	1 µL	2 µL
WGBS Adapter Dilution Buffer	49 µL	9 µL	3 µL	2 µL
Amount per reaction	2.5 µL	2.5 µL	2.5 µL	2.5 µL

3.1 For each sample, combine the following reagents (in this order) in the PCR tube or 8-tube strip:

Adenylated DNA (from Step 3)	20.5 µL
WGBS Ligation Mix (purple cap)	31.5 µL
WGBS Adapter (purple cap)	2.5 µL
TOTAL	**54.5 µL**

3.2 Mix thoroughly by pipetting.

3.3 Incubate on a thermocycler for 15 minutes at 22°C.

Clean-Up

3.4 Add **44 µL of AMPure XP Beads** to each sample and mix thoroughly by pipetting.

3.5 Incubate at room temperature for 5 minutes.

3.6 Place the tube on the DiaMag02 at room temperature for 5 minutes or until the supernatant appears clear.

3.7 Remove and discard clear supernatant. Take care not to disturb beads.

3.8 Add **200 µL of freshly prepared 80% ethanol** to each sample and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

3.9 Repeat previous step, for a total of 2 ethanol washes and ensure all ethanol has been removed.

3.10 Remove the tube from the DiaMag02 and let dry at room temperature for 3 minutes.

3.11 **NOTE**:Do not over dry the beads.

3.12 Resuspend dried beads with **52 µL of Resuspension Buffer**. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.

3.13 Incubate resuspended beads at room temperature for 2 minutes.

3.14 Place tube on DiaMag02 at room temperature for 5 minutes or until the sample appears clear.

3.15 Transfer **50 µL of clear sample** to new tube.

STEP 4

Gel-free Size Selection

 *30 minutes*

***NOTE**: Beads Size Selection has been re-optimized and the protocol option with gel size-selection has been removed. Researchers who still wish to size select libraries using agarose gel can contact us at info@diagenode.com and we will be happy to provide corresponding protocol and reagents.*

4.1 Add **28 µL of AMPure XP Beads** to each sample and mix thoroughly by pipetting.

4.2 Incubate at room temperature for 5 minutes.

4.3 Place the tube on the DiaMag02 at room temperature for 5 minutes or until the supernatant appears clear.

4.4 **DO NOT DISCARD CLEAR SAMPLE IN THIS STEP**. Transfer **76 µL of clear sample** to a new tube.

4.5 Add **12 µL of AMPure XP Beads** to each sample and mix thoroughly by pipetting

4.6 Incubate at room temperature for 5 minutes.

4.7 Place the tube on the DiaMag02 at room temperature for 5 minutes or until the supernatant appears clear.

4.8 Remove and discard clear supernatant. Take care not to disturb beads.

4.9 Add **200 µL of freshly prepared 80% ethanol** to each sample and incubate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

4.10 Repeat previous step, for a total of 2 ethanol washes and ensure all ethanol has been removed.

4.11 Remove the tube from the DiaMag02 and let dry at room temperature for 3 minutes.

***NOTE**: Do not over dry the beads.*

4.12 Resuspend dried beads with **36 µL of Resuspension Buffer**. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the tube.

4.13 Incubate resuspended beads at room temperature for 2 minutes.

4.14 Place the tube on DiaMag02 at room temperature for 5 minutes until the sample appears clear.

4.15 Transfer **33 µL of clear sample** to new 0.2 mL tube.

***NOTE**: If you wish to pause your experiment, the procedure may be safely stopped at this step and samples stored at -20°C. To restart, always thaw your frozen samples on ice before proceeding.*

STEP 5

Bisulfite conversion

 *4 hours*

5.1 Before the first use add **4 mL of 100 % ethanol** to 1 mL BS Wash Buffer concentrate. The BS Conversion Reagent supplied within this kit is a solid mixture and must be prepared prior to first use.

5.2 Add **790 µL of BS Solubilization Buffer** (black cap) and **300 µL of BS Dilution Buffer** (black cap) to the tube of BS Conversion Reagent.

5.3 Mix at room temperature with frequent vortexing or shaking for 10 minutes.

5.4 Add **160 µL of BS Reaction Buffer** (black cap) and mix an additional 1 minute.

***NOTE**: It is normal to see trace amounts of undissolved reagent in the BS Conversion Reagent. Each tube of BS Conversion Reagent is designed for 8 separate DNA treatments including an excess.*

***STORAGE**: The BS Conversion Reagent is light sensitive, so minimize its exposure to light. For best results, the BS Conversion Reagent should be used immediately following preparation. If not used immediately, the BS Conversion Reagent solution can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C but the conversion efficiency may be reduced. Stored BS Conversion Reagent solution must be warmed to 37°C, then vortexed prior to use.*

5.5 Add **117 µL of BS Conversion reagent** to 33 µL of sample from previous step, mix, centrifuge briefly and incubate in a thermocycler as indicated in Table 3.

Table 3.

Cycle Step	Temperature	Time	Cycles
Denaturation	95°C	1 minute	20
Conversion	60°C	10 minute	
Hold	4°C	∞	

5.6 Place BS Spin columns into the provided BS Collection Tubes.

5.7 Add **600 µL of BS Binding Buffer** into a BS Spin Column.

5.8 Load bisulfite-converted libraries into the BS Spin Columns containing the BS Binding Buffer. Close the cap and mix by inverting the column several times.

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

5.10 Add **100 µL of BS Wash Buffer** to each column. Centrifuge at full speed for 30 seconds.

5.11 Add **200 µL of BS Desulphonation Buffer** to each column and let stand at room temperature (20-30°C) for exactly 30 minutes. After the incubation, centrifuge at full speed for 30 seconds.

5.12 Add **200 µL of BS Wash Buffer** to each column. Centrifuge at full speed for 30 seconds. Add another **200 µL of BS Wash Buffer** and centrifuge for an additional 30 seconds.

5.13 Place each column into a 1.5 mL tube. Add **22 µL of BS Elution Buffer** (white cap) directly to the center of the column matrix and wait for 2 minutes. Centrifuge for 30 seconds at full speed to elute the DNA.

5.14 Elute one more time by transferring the eluate to the column's membrane. Wait 2 minutes, then centrifuge for 30 seconds at full speed.

***NOTE**: Proceed with the PCR amplification reaction quickly.*

STEP 6

PCR Amplification

1 hour

The table 4 can serve as a guideline in deciding the number of PCR cycles.

Table 4.

Input DNA	≤ 50 ng	100 ng	250 ng	500 ng	1 µg
Fragmented DNA	5-18 cycles	10-12 cycles	9-12 cycles	7-10 cycles	7-9 cycles

6.1 For each sample, combine the following reagents (in this order) in the PCR tube or 8-tube strip:

Bisulfite Converted Product (from Step 6)	20 µL
Nuclease free Water (white cap)	3 µL
MethylTaq Plus 2X Master Mix (green cap)	25 µL
WGBS Primer Mix (green cap)	2 µL
TOTAL	**50 µL**

6.2 Mix thoroughly by pipetting.

6.3 PCR Cycles:

10 min	95°C	
30 sec	98°C	
30 sec	65°C	Repeat 9 - 18 cycles**
45 sec	72°C	
7 min	72°C	

***PCR cycles will vary depending on the amount of starting material and quality of your sample. Further optimization may be necessary. Always use the least number of cycles possible.*

6.4 Add **40 µL of AMPure XP Beads** to each sample and mix thoroughly by pipetting.

6.5 Incubate at room temperature for 5 minutes.

6.6 Place the tube on the DiaMag02 at room temperature for 5 minutes or until the supernatant appears clear.

6.7 Remove and discard clear supernatant. Take care not to disturb beads. Some liquid may remain in tubes.

6.8 Add **200 µL of freshly prepared 80% ethanol** to each magnetic bead pellet and incubate plate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

6.9 Repeat previous step, for a total of 2 ethanol washes and ensure all ethanol has been removed.

6.10 Remove the tube from the DiaMag02 and let dry at room temperature for 3 minutes.

6.11 Resuspend dried beads with **52 µL of Resuspension Buffer**. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the tube.

6.12 Incubate resuspended beads at room temperature for 2 minutes.

6.13 Place tube on DiaMag02 for 5 minutes or until the sample appears clear.

6.14 Transfer **50 µL of clear sample** to new tube.

6.15 Add **40 µL of AMPure XP Beads** to each sample and mix thoroughly by pipetting.

6.16 Incubate at room temperature for 5 minutes.

6.17 Place the tube on the DiaMag02 at room temperature for 5 minutes or until the supernatant appears clear.

6.18 Remove and discard clear supernatant. Take care not to disturb beads. Some liquid may remain in tubes.

6.19 Add **200 µL of freshly prepared 80% ethanol** to each magnetic bead pellet and incubate at room temperature for 30 seconds. Carefully, remove ethanol by pipette.

6.20 Repeat previous step, for a total of 2 ethanol washes and ensure all ethanol has been removed.

6.21 Remove the tube from the DiaMag02 and let dry at room temperature for 3 minutes.

6.22 Resuspend dried beads with **16 µL of Resuspension Buffer**. Mix thoroughly by pipetting. Ensure beads are no longer attached to the side of the well.

6.23 Incubate resuspended beads at room temperature for 2 minutes.

6.24 Place tube on DiaMag02 for 5 minutes or until the sample appears clear.

6.25 Transfer **15 µL of clear sample** to a new tube.

6.26 Examine your library with a fluorometer and check the size using an Agilent Bioanalyzer or 2% agarose gel (See Examples in Figure 2).

Example of results

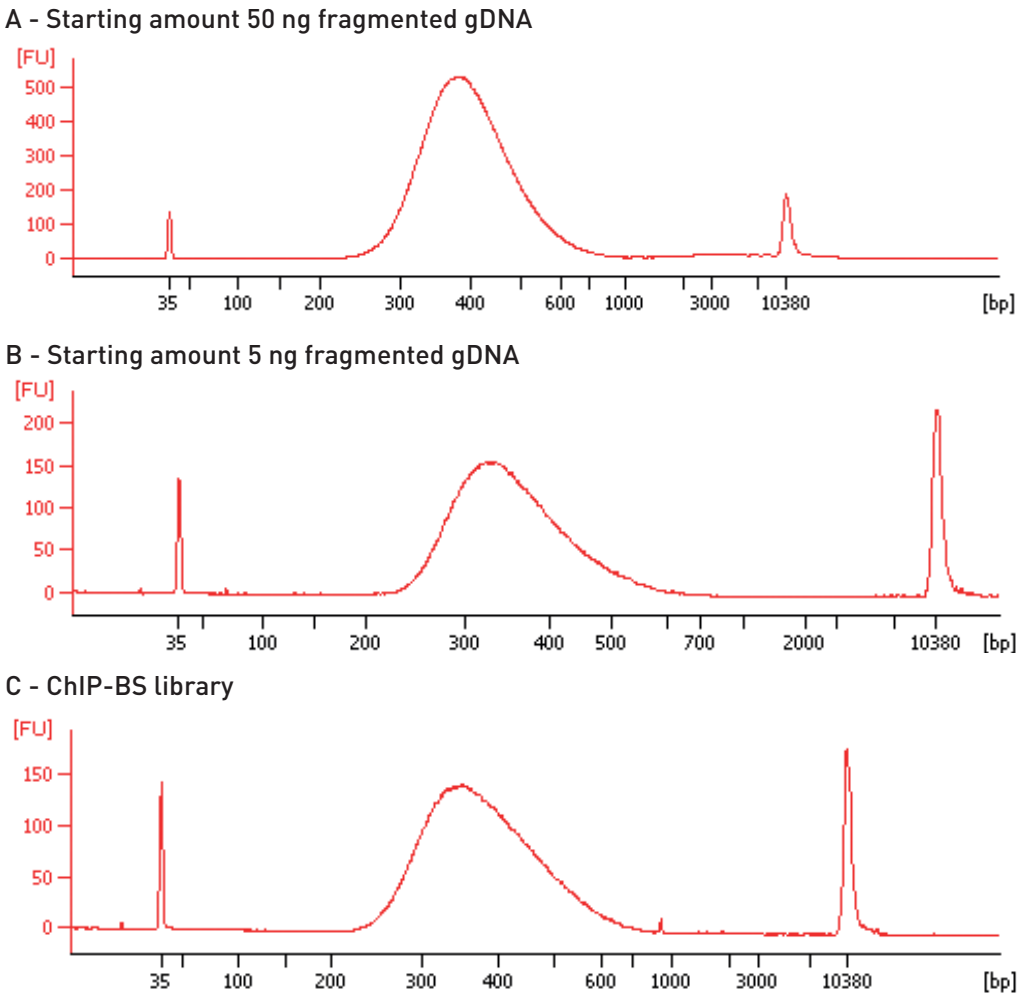


Figure 2. Bioanalyzer profile of different libraries obtained with the Premium WGBS Kit.

A. 50 ng of fragmented genomic DNA was used. A total of 18 cycles of PCR were performed after bisulfite conversion. 1 µL of the resulting library was diluted 5-fold and run on an Agilent High Sensitivity DNA chip.

B. 5 ng of fragmented genomic DNA was used. A total of 18 cycles of PCR were performed after bisulfite conversion. 1 µL of the resulting library was run on an Agilent High Sensitivity DNA chip.

C. 50 ng of ChIP'ed DNA was used. The Chromatin Immunoprecipitation was performed against the H3K27me3 histone mark, using the Diagenode's iDeal ChIP-seq Kit for Histones (Cat. No. C01010050 and C01010051). A total of 18 cycles of PCR were performed after bisulfite conversion. 1 µL of the resulting library was diluted 2.5-fold and run on an Agilent High Sensitivity DNA chip.

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