diagenode

A Hologic Company

Premium RRBS Kit V2

Reduced Representation Bisulfite Sequencing for Illumina® Platforms

Cat. No. C02030036 (24 rxns)
C02030037 (96 rxns)

USER GUIDE

Version 2 04_2024

Please read this manual carefully
before starting your experiment

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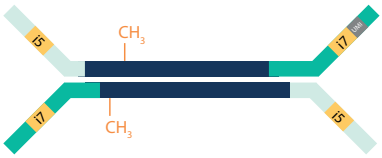
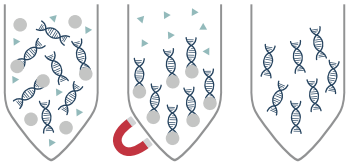
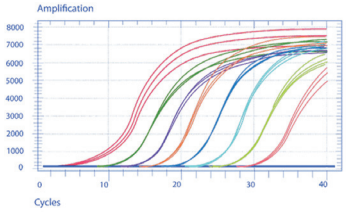
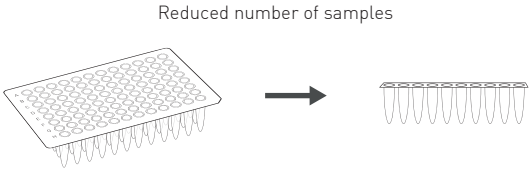
STEP 2		Library preparation	Day 2 2h15
STEP 3		Size selection	Day 2 1h
STEP 4		Quantification	Day 2 1h30
STEP 5		Sample pooling	Day 3 30 min
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Legend

DNA

Y adapters

i5/i7 Unique dual indexes

Unique molecular identifier

Introduction

DNA methylation is a key epigenetic mechanism with important regulatory functions in biological processes such as genomic imprinting, control of transcription, embryonic development, X-chromosome inactivation, chromosome stability, and carcinogenesis. Its study in diseased cells is of growing interest for new diagnostic and therapeutic methods development.

DNA methylation primarily takes place by the addition of a methyl group to the C5 position of a cytosine nucleotide, which occurs mainly in the context of CpG dinucleotides, forming 5-methylcytosine (5-mC). The most common technique used in DNA methylation studies remains bisulfite conversion prior to analysis. Whole-genome bisulfite sequencing (WGBS) is the method of choice for obtaining a comprehensive DNA methylation profiling, evaluating the methylation patterns of nearly every CpG site of the entire genome. However, a high read depth is required to reliably determine methylation status. When working on organisms with a large genome size, this can lead to high costs for sequencing. As an alternative, one can focus the detection of DNA methylation to a specific subset of the genome, thereby reducing the data volume of your experiment and subsequently the cost.

Reduced Representation Bisulfite Sequencing (RRBS) offers a **cost-effective, focused solution** to perform genome-scale DNA methylation analysis at the **single nucleotide level** in any vertebrate species. The fundamental idea of RRBS is to get a "reduced representation" of the genome, with a focus on **CpG islands**. By cutting the genome using the restriction MspI enzyme (5' -CCGG- 3' target sites), followed by size selection, the DNA sample is enriched with biologically relevant CpG-rich regions (including promoters and CpG islands) in which regulatory DNA methylation marks are typically found.

Functional CpG-rich regions are often unmethylated, which is an important feature in the regulation of gene expression. By enriching and sequencing those CpG-rich regions, RRBS provides a cost-effective method to collect **meaningful DNA methylation data**, while reducing the amount of sequencing needed, leading to a substantially decreased cost. Diagenode's Premium RRBS Kit V2 has been specifically developed and

optimized to generate RRBS libraries from the **lowest DNA amounts** (down to **25 ng of genomic DNA**) and secure **high-quality NGS data** for DNA methylation analysis.

One distinguishing feature of Premium RRBS kits is its support for **early sample pooling** prior to bisulfite conversion, which reduces the handling time and cost per sample. The workflow allows processing of **96 samples per experiment**, enabling studies of large cohorts. New additions to the Premium RRBS set of features are the use of:

- **Unique Dual Indexes (UDI)** to mitigate errors introduced by read misassignment, including index hopping frequently observed with patterned flow cells such as Illumina's NovaSeq platform.
- **Unique Molecular Identifiers (UMI)** to identify and remove PCR duplicates from your data and recover an accurate counting. This feature is especially appreciable in case of low DNA amount analysis.

Diagenode's Premium RRBS kits are widely used by services providers as well as research laboratories worldwide thanks to its numerous benefits:

- Size selection has been optimized to keep small fragments of interest and to remove adapter dimers, resulting in a better coverage.
- The pooling strategy allows you to manipulate fewer tubes, providing an easier-to-handle and cost-effective protocol.
- The bisulfite conversion protocol has been improved to decrease DNA degradation while keeping a highly efficient conversion of unmethylated cytosines.
- Positive and negative spike-in controls are included for the monitoring of bisulfite conversion efficiency.
- The minimum number of amplification cycles needed for each pool is determined to avoid amplification biases and limit the level of PCR duplicates. Our MethylTaq Plus 2X master mix was developed to amplify bisulfite converted DNA with high efficiency, and reduces the number of PCR cycles required.

Kit Materials

The Premium RRBS Kit V2 contains reagents necessary for processing of 24 or 96 individual samples at steps 1 to 5, and of 5 or 20 pools at steps 6 and 7, depending on the kit size (24 rxns and 96 rxns, respectively).

Table 1a. Components of the Premium RRBS Kit V2 – Box 1/2

Component	Cap color	Qty (24 rxns)	Qty (96 rxns)	Storage
Enzyme buffer	Purple	100 µl	330 µl	-20°C
Restriction enzyme	Purple	35 µl	110 µl	-20°C
Ends preparation enzyme	Blue	35 µl	110 µl	-20°C
dNTP mix	Blue	35 µl	110 µl	-20°C
Unmethylated spike-in control	Blue	35 µl	110 µl	-20°C
Methylated spike-in control	Blue	35 µl	110 µl	-20°C
Adapter dilution buffer	Red	100 µl	144 µl	-20°C
Ligation buffer	Red	1300 µl	4400 µl	-20°C
Ligase	Red	35 µl	110 µl	-20°C
Primer mix	Green	50 µl	170 µl	-20°C
MethylTaq Plus 2X master mix	Green	180 µl	600 µl	-20°C
100x SYBR	Brown	5 µl	5 µl	-20°C
Resuspension buffer	White	1000 µl	4500 µl	-20°C or + 4°C
Nuclease-free water	/	1390 µl	4800 µl	-20°C or + 4°C

Table 1b. Components of the Premium RRBS Kit V2 – Box 2/2

Component	Cap color	Qty (24 rxns)	Qty (96 rxns	Storage
DNA binding buffer	Yellow	3000 µl	12000 µl	RT
DNA wash buffer w/o ethanol	Yellow	600 µl	1600 µl	RT
BS Conversion reagent	Brown	1 tube	2 µl	RT
BS Dilution buffer	Black	300 µl	600 µl	RT
BS Solubilization buffer	Black	790 µl	1600 µl	RT
BS Reaction buffer	Black	160 µl	320 µl	RT
BS Binding buffer	/	3000 µl	12000 µl	RT
BS Wash buffer w/o ethanol	/	600 µl	2000 µl	RT
BS Desulphonation buffer	/	1000 µl	4000 µl	RT
BS Elution buffer	White	110 µl	440 µl	RT
DNA Spin columns	/	10 columns	40 columns	RT
DNA Collection tubes	/	10 tubes	40 tubes	RT

Storage

The components should be stored at temperatures indicated in Tables 1a & 1b.

Required Materials Not Provided

Methyl UDI-UMI Adapters

Specific Premium Methyl UDI-UMI Adapters were designed with UDI and UMI and validated for Premium RRBS experiments. These adapters are **not included** in this kit and should be ordered separately to proceed with RRBS library preparation. Please choose the format that matches your multiplexing needs among the compatible references:

Table 2. Premium Methyl UDI-UMI Adapters Modules

Methyl UDI-UMI Adapters	Format	Cat. No.
Premium Methyl UDI-UMI Adapters Module – Set A	24 UDI-UMI, 24 rxns	C02030040
Premium Methyl UDI-UMI Adapters Module – Set B	24 UDI-UMI, 24 rxns	C02030041
Premium Methyl UDI-UMI Adapters Module - 96 rxns	96 UDI-UMI, 96 rxns	C02030042

All Premium Methyl UDI-UMI Adapters are supplied at 100 nM in a volume sufficient for 1 rxn (5 µl). Please note that depending on your DNA starting amount, dilution of the indexed-adapters might be needed. Refer to the protocol for instructions.

The sequences of Premium Methyl UDI-UMI adapters are listed in the section: Premium Methyl UDI-UMI Adapters Sequences.

General equipment and Reagents

- Gloves to wear at all steps
- Autoclaved tips
- Nuclease-free tubes: 0.2 ml, 1.5 ml, 15 ml
- 96-well plates, 96-well qPCR plates and adapted adhesive plate seals
- Multichannel pipettes (12 channels)
- 12-tube strips
- Racks for 0.2 ml tube strips
- Centrifuge for 0.2 ml tube strips
- Thermocycler and qPCR thermocycler

- qPCR reagents (SybrGreen MasterMix)
- Centrifuge for 96-well plates
- Centrifuge for 1.5 and 15 ml tubes
- 96-well plate magnetic rack

Size selection & post-PCR libraries purification

- Agencourt® AMPure® XP Beads (Beckman Coulter, A63881)
- 100% ethanol
- Nuclease-free water

Library quality control

- Sizing equipment such as BioAnalyzer (Agilent) or Fragment Analyzer (Agilent) and their associated high sensitivity kits
- Fluorescence-based dsDNA quantification assay equipment such as the Qubit® Fluorometer (Thermo Fisher Scientific) and Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Q32851)

Additional supplies (included and available separately)

Component	Cat. No.	Format
Bisulfite conversion reagent for RRBS	C02030035	8 rxns
DNA methylation control package V2	C02040019	48 rxns
MethylTaq Plus 2X Master Mix	C09010012	135 µl
MicroChIP DiaPure columns	C03040001	50 rxns

Optional supplies (not included and available separately)

Component	Cat. No.	Format
XL GenDNA Extraction Module	C03030020	6 rxns

Remarks Before Starting

Early sample pooling strategy

The Premium RRBS protocol has been optimized for a high number of samples and allows processing of up to 96 samples per experiment. The workflow includes an early sample pooling prior to bisulfite conversion, which reduces the handling time and cost per sample. During next-generation sequencing, several samples are usually processed in one lane, because the number of reads is too high for only one sample. The pooling step in the Premium RRBS protocol permits to group together samples that will be sequenced in the same lane early in their treatment. The number of samples that can be pooled together depends on several parameters:

1. The number of reads per lane that the sequencer is providing. This is different for every sequencer and flowcell model.

2. The number of reads that are needed for each sample in order to properly cover the CpG islands. For standard analysis of human or mouse samples, we recommend 30-40 million of raw reads per sample. This varies according to the size of the genome studied.

3. The mapping efficiency for the genome of interest. After sequencing, the reads obtained will be aligned to the reference genome. If the genome is not well-known, or contains a lot of repeats, a substantial percentage of reads may be lost at this step, which will reduce coverage.

4. The number of barcodes available. To distinguish the samples that are mixed in one sequencing lane, they have to carry different barcodes.

In general, we recommend pooling samples by a minimum of 5 in order to decrease the number of PCR cycles needed for library amplification and thus the level of PCR duplicates in the sequencing data. For human and mouse samples to be sequenced on NovaSeq 6000 System – Flow Cell Type SP, we recommend pooling 10 samples per lane.

Nevertheless it is possible to process some samples individually without pooling, or pools of up to 24 samples as well, as long as the total number of samples or pools do not exceed 5 for the 24 rxns kit and 20 for the 96 rxns kit.

Genomic DNA extraction

The quality of the gDNA to be used in a RRBS experiment is important. We recommend using Diagenode's XL GenDNA Extraction module (C03030020) for the extraction and purification of genomic DNA from cultured cells. gDNA from other source than cultured cells can also be used for RRBS as long as gDNA is of good quality (high integrity, not fragmented) and sufficient quantity (minimum 25 ng per sample). We recommend checking for gDNA degradation by analysis of a small aliquot of each sample on a 0.8 % agarose gel or on a Fragment Analyzer (Agilent). For optimal results the gDNA must be larger than 10 kb.

For the quantification of double-stranded genomic DNA, a fluorescence-based assay such as the Qubit High Sensitivity assay (Thermo Fischer Scientific) must be used, since spectrophotometric measurements (e.g. NanoDrop) might overestimate the amount of dsDNA.

NOTES:

- *When the number of cells is limited, a phenol-chloroform extraction can be used.*
- *Regardless the choice of DNA extraction protocol, proteinase K digestion is mandatory.*
- *Do not use Trizol during your DNA extraction as it inhibits the enzymatic digestion.*
- *Do not vortex high molecular weight DNA as this might lead to fragmentation. Mix by pipetting.*

Starting material

The Premium RRBS V2 protocol has been validated for gDNA starting amounts ranging **from 25 ng to 100 ng**. DNA samples should be dissolved in 26 µl of nuclease-free water before starting the protocol.

Quantitative PCR

The protocol contains two qPCR steps. The reagents are not provided for the first qPCR, so that every researcher can use their regular SybrGreen mastermix that fits their qPCR device. For the second qPCR, the required reagents are provided (MethylTaq Plus 2X Master Mix and 100X SYBR). For a good estimate of the number of PCR cycles to apply during library

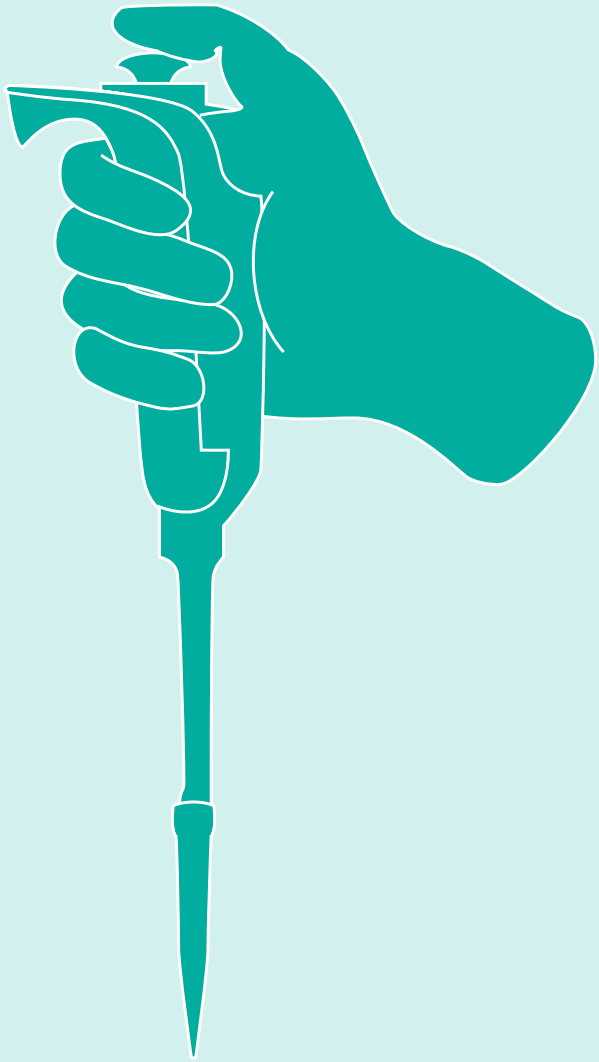
amplification, it is highly recommended to use the MethylTaq Plus 2X Master Mix for both this qPCR step and the library amplification. MethylTaq Plus 2X Master Mix is optimized for amplification of bisulfite-converted DNA involved in these protocol steps.

Quality controls

The Premium RRBS Kit V2 contains one methylated and one unmethylated DNA spike-in controls to monitor the efficiency of bisulfite conversion during downstream bioinformatic analysis (see p40).

We recommend to add one of each spike-in to every sample (step 2.1) to control for under- and over-conversion during sodium bisulfite treatment.

PROTOCOL



STEP 1

Enzymatic digestion

1 Day 1 30 min (Overnight incubation)

1.1 Prepare your samples in a **total volume of 26 µl in nuclease-free water** in a 96-well plate.

- Start loading your samples in well A01 and proceed in rows

CAUTION: *It is necessary to proceed in rows when distributing the samples as the adapters will be distributed the same way. This will help to limit the indexes incompatibilities between samples of the same project.*

1.2 Prepare the **Digestion Mix** in a 1.5 ml nuclease-free tube as described in the following table and mix by pipetting.

	Mix for # samples (µl)								
Component	**Volume/ reaction**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Enzyme buffer ●	3 µl	60	96	150	192	225	264	300	330
Restriction enzyme ●	1 µl	20	32	50	64	75	88	100	110
Total volume	**4 µl**	**80**	**128**	**200**	**256**	**300**	**352**	**400**	**440**

NOTE: *If processing any other number of samples, calculate the total volume of each reagent by multiplying the volume needed per reaction by the number of samples. Include 15% bonus volume if you have less than 12 samples and you distribute the Digestion Mix directly to your samples in the 96-well plate (skipping step 1.3). Include 20-25% bonus volume if you have 12 samples or more and proceed to step 1.3 for ease of distribution with the multichannel pipette.*

1.3 Transfer the volume of **Digestion Mix** indicated below to each tube of 12-tube strips. This will allow for multichannel pipette use.

	Mix for # samples (µl)						
	24	**36**	**48**	**60**	**72**	**84**	**96**
Volume	10	16	20	24	28	32	36

NOTE: *This step is not needed when processing less than 24 samples.*

1.4 Distribute **4 µl of Digestion Mix** to each sample of the 96-well plate using a multichannel pipette.

1.5 Mix by pipetting up and down 10 times.

1.6 Seal the 96-well plate, spin briefly, place it in a thermocycler with heated lid on (105°C) and run the following program overnight:

Temperature	Time
37°C	12 h
8°C	Hold

STEP 2

Library preparation

2 Day 2 2 h 15 min

2.1 Prepare the **Ends Preparation Mix** in a 1.5 ml nuclease-free tube as described in the following table and mix by pipetting.

CAUTION: *When using less than 100 ng of gDNA, proportionally dilute the unmethylated and methylated spike-in controls in nuclease-free water: e.g. for 50 ng gDNA use controls diluted 1/2 ; for 25 ng gDNA use controls diluted 1/4. For 100 ng of gDNA no dilution is needed.*

		Mix for # samples (µl)							
Components	**Volume/ reaction**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Ends preparation enzyme	1 µl	20	32	50	64	75	88	100	110
dNTP mix	1 µl	20	32	50	64	75	88	100	110
Unmethylated spike-in control	1 µl	20	32	50	64	75	88	100	110
Methylated spike-in control	1 µl	20	32	50	64	75	88	100	110
Total volume	**4 µl**	**80**	**128**	**200**	**256**	**300**	**352**	**400**	**440**

NOTE:

- *If processing any other number of samples, calculate the total volume of each reagent by multiplying the volume needed per reaction by the number of samples. Include 15% bonus volume if you have less than 12 samples and you distribute the Ends Preparation Mix directly to your samples in the 96-well plate (skipping step 2.2). Include 20-25% bonus volume if you have 12 samples or more and proceed to step 2.2 for ease of distribution with the multichannel pipette.*

- *The amount of spike-in control has been calculated to have a good coverage when several samples are pooled together. If you plan to process single samples or pools smaller than 4 we recommend adding 2 µl of each spike-in control instead of 1 µl.*

2.2 Transfer the **volume of Ends Preparation Mix** indicated below to each tube of 12-tube strips.

	Mix for # samples (µl)						
	24	**36**	**48**	**60**	**72**	**84**	**96**
Volume	10	16	20	24	28	32	36

NOTE: *This step is not needed when processing less than 24 samples.*

2.3 Distribute **4 µl of Ends Preparation Mix** to each sample of the 96-well plate using a multichannel pipette.

2.4 Mix by pipetting up and down 10 times.

2.5 Seal the 96-well plate, spin briefly, place it in a thermocycler with heated lid on (105°C) and run the following program for 1 h:

Temperature	Time
30°C	20 min
37°C	20 min
75°C	20 min
8°C	Hold

CAUTION: *Do not stop at this step and proceed directly to adapter ligation.*

2.6 Thaw the **Ligation Buffer** ● about 15 min before the ends preparation reaction finishes.

2.7 Thaw the **Premium Methyl UDI-UMI Adapters** ● and centrifuge them briefly.

2.8 According to the DNA starting amounts, dilute the **Premium Methyl UDI-UMI Adapters** ●, if necessary, with the **Adapter Dilution Buffer** ● in a 0.2 ml nuclease-free tube as described in the following table and mix by pipetting.

DNA starting amount	Adapter dilution	Working adapter concentration
100 ng – 50 ng	No dilution	100 nM
<50 ng – 25 ng	3/4	75 nM

NOTE: *For example, to prepare 6 µl of Premium Methyl UDI-UMI Adapter at 75 nM, take 4.5 µl of Premium Methyl UDI-UMI Adapter at 100 nM and add 1.5 µl Adapter Dilution Buffer.*

2.9 Add **5 µl of the working Premium Methyl UDI-UMI Adapter** to each sample of the 96-well plate.

NOTE: *There is no need to decide now which samples will be pooled together, as long as each sample has a different indexed Premium Methyl UDI-UMI Adapter. If the same set of indexes will be used several times, distribute them in rows in order to minimize the risk to have two times the same index in samples from the same project.*

2.10 Prepare the **Ligation Mix** in a 15 ml or 1.5 ml nuclease-free as described in the following table and mix by pipetting.

		Mix for # samples (µl)							
Components	**Volume/ reaction**	**12**	**24**	**36**	**48**	**60**	**72**	**84**	**96**
Ligation Buffer	40 µl	800	1280	2000	2560	3000	3520	4000	4400
Ligase	1 µl	20	32	50	64	75	88	100	110
Total volume	**41 µl**	**820**	**1312**	**2050**	**2614**	**3075**	**3608**	**4100**	**4510**

NOTE: *If processing any other number of samples, calculate the total volume of each reagent by multiplying the volume needed per reaction by the number of samples. Include 15% bonus volume if you have less than 12 samples and you distribute the Ligation Mix directly to your samples in the 96-well plate (skipping step 2.12). Include 20-25% bonus volume if you have 12 samples or more and proceed to step 2.12 for ease of distribution with the multichannel pipette.*

2.11 Transfer the volume of **Ligation Mix** indicated below to each tube of 12-tube strips.

NOTE: *This step is not needed when processing less than 24 samples.*

NOTE: *When processing 60 samples or more, transfer the Ligation Mix in two strips due to maximum well capacity.*

	Mix for # samples (µl)						
	24	**36**	**48**	**60**	**72**	**84**	**96**
Volume/tube in the 1st strip	103	144	185	130	134	170	175
Volume/tube in the 2nd strip	0	0	0	90	134	130	175

2.12 Distribute **41 µl of Ligation Mix** to each sample of the 96-well plate using a multichannel pipette. The resulting reaction volume is 80 µl.

2.13 Mix by pipetting up and down 10 times.

2.14 Seal the 96-well plate, spin briefly, place it in a thermocycler with heated lid on (105°C) and run the following program for 30 min.

Temperature	Time
25°C	20 min
65°C	10 min
8°C	Hold

STEP 3

Size selection

2 Day 2 1 h

3.1 Carefully resuspend the **AMPure XP beads** at room temperature by shaking and light vortexing until no pellet is visible at the bottom of the container.

3.2 Add **60 µl of AMPure XP Beads** to each sample of the 96-well plate and mix by pipetting up and down at least 10 times until the mixture is homogenous.

3.3 Incubate at room temperature for at least 15 min.

3.4 Thaw the Resuspension Buffer at room temperature.

3.5 Place the 96-well plate on a 96-well magnetic rack and wait until the beads are completely bound to the magnet (~5 min).

3.6 Carefully remove and discard the supernatant using a multichannel pipette without disturbing the beads that contain the desired DNA fragments.

3.7 Wash the beads pellets of the first row twice as follows:

- With the 96-well plate on the 96-well magnetic rack, add **100 µl of freshly prepared 80% Ethanol** without disturbing the beads pellets and wait for 5 sec.
  CAUTION: *Do not leave the beads pellets longer than 5 sec in 80% Ethanol as it may already elute DNA from the beads.*
- Carefully remove and discard the supernatant using a multichannel pipette without disturbing the beads pellets.
- Be sure to remove all visible liquid after the second wash.
- Allow the beads pellets to air-dry 3 min.

NOTE: *When processing 13 samples or more, perform the washes of the second row while the first row is air-drying and repeat the process for as many rows as needed.*

3.8 Transfer the volume of Resuspension Buffer indicated below to each tube of 12-tube strips.

	Buffer for # samples (µl)						
	24	**36**	**48**	**60**	**72**	**84**	**96**
Volume	58	86	115	144	173	201	230

NOTE: *This step is not needed when processing less than 24 samples.*

3.9 Distribute **25 µl of Resuspension Buffer** to each sample of the 96-well plate with a multichannel pipette, vortex carefully to resuspend the beads and spin down briefly.

3.10 Incubate at room temperature for 5 min out of the magnetic rack to elute DNA.

3.11 Place the 96-well plate on the 96-well magnetic rack and wait until the beads are completely bound to the magnet (~5 min).

3.12 Without disturbing the beads pellets, carefully aspirate and transfer the supernatants to a new 96-well plate. Discard the beads.

NOTE: *This plate can be conserved at -20°C for several days.*

STEP 4

Quantification

2 Day 2 1 h 30 min

4.1 Prepare the **Quantification Mix** in a 2 ml nuclease-free tube. An example using a 2X qPCR Master Mix with SybrGreen (not provided) is indicated in the following table. If your qPCR Master Mix is provided at another concentration, adapt the volumes for Quantification Mix preparation. Perform the qPCR in duplicates.

		Mix for # reactions (µl)							
Components	**Volume/ reaction**	**24**	**48**	**72**	**96**	**120**	**144**	**168**	**192**
2X qPCR Master Mix	5 µl	144	288	432	576	720	864	1008	1152
Primer Mix	0.5 µl	14.4	28.8	43.2	57.6	72	86.4	100.8	115.2
Nuclease-free Water	3.5 µl	100.8	201.6	302.4	403.2	504	604.8	705.6	806.4
Total volume	**9 µl**	**259.2**	**518.4**	**777.6**	**1036.8**	**1296**	**1555.2**	**1814.4**	**2073.6**

4.2 Transfer the volume of **Quantification Mix** indicated below to each tube of 12-tube strips. Each library will be quantified in duplicates and the average Ct value will be used to calculate the volumes for pooling.

NOTE: *You can prepare additional qPCR reactions for a positive control (e.g. a previously tested library) and a negative control (water).*

	Mix for # reactions (µl)							
	24	**48**	**72**	**96**	**120**	**144**	**168**	**192**
Volume	21	41	62	83	104	124	145	165.6

4.3 Distribute **9 µl of Quantification Mix** to 2 wells per sample of a new 96-well qPCR plate using a multichannel pipette.

4.4 Add **1 µl of sample** to each well of the 96-well qPCR plate.

NOTE: *If the multichannel pipette you are using is not accurate enough to pipette 1 µl of sample you can perform an intermediary dilution: Mix in a new plate 3 µl of each sample and 6 µl of nuclease-free water. Then use 3 µl of this dilution for the qPCR and decrease the amount of water per reaction in the quantification mix down to 1.5 µl.*

4.5 Seal the 96-well plate, mix by vortexing, centrifuge briefly and place it in a qPCR machine. Run the following program for 1 h.

Step	Temperature	Time	Cycles
Initial denaturation*	98°C	3 min	1
Denaturation	95°C	15 sec	25
Annealing	60°C	30 sec	
Extension	72°C	30 sec	

** Please check carefully supplier's recommendations about Taq polymerase activation time and temperature.*

NOTE: *When processing more than 48 samples two qPCR plates are needed. You can keep the second plate at 4°C during the first run.*

NOTE: *You can add a melting curve step at the end of the qPCR.*

STEP 5

Sample pooling

3 Day 3 30 min

NOTE: *At this step, all samples that will be sequenced in the same lane can be pooled in one tube. Please refer to the paragraph "Early sample pooling strategy" (p12) to determine the optimal number of samples per pool to be sequenced together.*

The pooling protocol uses the Ct values from the qPCR at step 4 to pool together samples with similar concentration and to calculate the volumes of each library to mix in order to have the same amount of each sample in the pool.

We propose 2 options to determine the pooling strategy:

For assisted calculations, get access to our online Software for Intelligent Pooling (SIP), download the template "Template_SIP_RRBS.xlsx", fill in your qPCR results in it and follow the step-by-step instructions.
https://diagenode.shinyapps.io/RRBS_SIP/

- For manual calculations, see our Additional protocol – Manual Calculation of Volumes for Pooling p44 and follow the step-by-step instructions using Microsoft Excel.

5.1 Pool combinations of libraries and nuclease-free water in new 1.5 ml microcentrifuge tubes according to the calculated volumes.

NOTE: *There is a "check" column in the output file from SIP that you can fill every time you pipette one sample, to make sure not to forgot one well.*

5.2 Add **5 volumes of DNA Binding Buffer** ● to **1 volume of Pooled Libraries** from step 5.10. (e.g. add 600 µl of DNA Binding Buffer to 120 µl of Pooled Libraries).

5.3 Mix briefly by inverting the closed tubes.

5.4 Place **DNA Spin Columns** in **DNA Collection Tubes**.

5.5 Transfer the **Pooled Libraries** from step 5.2 in the **DNA Spin Columns**.

NOTE: *If the volume of the pooled libraries with DNA Binding Buffer is higher than 720 µl, load only half of the sample on the DNA Spin Column.*

5.6 Centrifuge at ≥ 10,000 x g for 30 sec. Discard the flow-through.

NOTE: *If the volume of the pooled libraries with DNA Binding Buffer is higher than 720 µl, repeat steps 5.5 and 5.6. loading the second half of the sample on the same column.*

5.7 When using the **DNA Wash Buffer** for the first time, add **4 volumes of 100 % ethanol** to **1 volume of DNA Wash Buffer** (dilution 1/5), as indicated on the label.

STORAGE: *After ethanol addition, the DNA Wash Buffer can be stored at room temperature.*

5.8 Add **200 µl of DNA Wash Buffer** to each column.

5.9 Centrifuge at ≥ 10,000 x g for 30 sec. Discard the flow-through.

5.10 Repeat steps 5.8 and 5.9. Discard the flow-through.

5.11 Centrifuge again at ≥ 10,000 x g for 1 min to dry column filters.

5.12 Transfer **DNA Spin Columns** to new 1.5 ml microcentrifuge tubes. Discard **DNA Collection Tubes**.

5.13 Add **36 µl of Resuspension Buffer** to each column and incubate for 2 min.

5.14 Centrifuge at ≥ 10,000 x g for 30 sec to elute the DNA.

5.15 Transfer the eluted DNA back on its column filter and incubate for 2 min.

5.16 Centrifuge at ≥ 10,000 x g for 30 sec to elute again the DNA.

STEP 6

Bisulfite conversion

3-4 Day 3-4 Overnight incubation + 1 h 30 min

CAUTION: *The BS Conversion Reagent has to be manipulated under a fume hood.*

6.1 The **BS Conversion Reagent** provided in the kit is a solid mixture and must be prepared before use. Add **790 µl of BS Solubilization Buffer ●** and **300 µl of BS Dilution Buffer ●** to a tube of **BS Conversion Reagent.**

6.2 Mix at room temperature with frequent vortexing or shaking for 10 min.

6.3 Add **160 µl of BS Reaction Buffer ●** to the tube of **BS Conversion Reagent** and mix at room temperature for 1 min.

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. Otherwise, it can be stored at -20°C/-4°F up to 1 month and re-used maximum once, without guarantee on the conversion efficiency. If needed, Bisulfite conversion reagent for RRBS (C02030035) can be purchased separately.*

NOTE: *It is normal to see trace amounts of undissolved reagent in the BS Conversion Reagent.*

6.4 Transfer **33 µl of each library pool** from step 5.14 in new tube strips (or in a new 96-well plate).

6.5 Add **117 µl of mixture of BS Conversion Reagent** to each library pool.

6.6 Mix the tube, centrifuge briefly and place it in a thermocycler with heated lid on (105°C). Run the following program for 4 h.

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

NOTE: *Bisulfite conversion can be performed overnight.*

6.7 Place **DNA Spin Columns** in **DNA Collection Tubes.**

6.8 Add **600 µl of BS Binding Buffer** in each column.

6.9 Transfer the **Bisulfite-converted Library Pools** from step 6.6 into the columns.

6.10 Mix briefly by inverting the closed columns.

6.11 Centrifuge at ≥ 10,000 x g for **30 sec**. Discard the flow-through.

6.12 When using the BS Wash Buffer for the first time, add **4 volumes of 100 % ethanol** to **1 volume of BS Wash Buffer** (dilution 1/5), as indicated on the label.

STORAGE: *After ethanol addition, the BS Wash Buffer can be stored at room temperature.*

6.13 Add **100 µl of BS Wash Buffer** to each column.

6.14 Centrifuge at ≥ 10,000 x g for 30 sec.

6.15 Add **200 µl of BS Desulphonation Buffer** to each column.

6.16 Incubate at room temperature for exactly 30 min.

6.17 Centrifuge at ≥ 10,000 x g for 30 sec. Discard the flow-through.

6.18 Add **200 µl of BS Wash Buffer** to each column.

6.19 Centrifuge at ≥ 10,000 x g for 30 sec.

6.20 Repeat steps 6.18 and 6.19. Discard the flow-through.

6.21 Transfer the **DNA Spin Columns** into a new 1.5 ml nuclease-free tube. Discard the **DNA Collection Tubes.**

6.22 Add **22 µl of BS Elution Buffer** to each column and incubate for 2 min.

6.23 Centrifuge at ≥ 10,000 x g for 30 sec to elute the DNA.

6.24 Transfer the eluted DNA back on its column filters and incubate for 2 min.

6.25 Centrifuge at ≥ 10,000 x g for 30 sec to elute again the DNA.

NOTE: *Bisulfite-converted DNA is highly unstable and we recommend proceeding with the library amplification as soon as possible.*

STEP 7

Library amplification

4 Day 4 3 h

DETERMINATION OF THE OPTIMAL NUMBER OF PCR CYCLES

7.1 Prepare the **Quantification Mix** in a 1.5 ml nuclease-free tube.

NOTE: *A single qPCR per library pool is sufficiently accurate, there is no need to perform it in duplicate.*

		Mix for # samples (µl)			
Components	**Volume/reaction**	**2**	**4**	**8**	**16**
MethylTaq Plus 2X Master Mix	5 µl	12.5	22.5	42.5	82.5
Primer Mix	0.5 µl	1.3	2.3	4.3	8.3
Nuclease-free Water	3.4 µl	8.4	15.2	28.8	56
100X SYBR*	0.1 µl	0.3	0.5	0.9	1.7
Total volume	**9 µl**	**22.5**	**40.5**	**76.5**	**148.5**

** If the volume of 100X SYBR is too small for pipetting you can dilute it 10X in nuclease-free water and add 1 µl of 10X SYBR per reaction. Adjust accordingly the volume of nuclease-free water in the mix, down to 2.5 µl.*

7.2 Transfer **9 µl of the Quantification Mix** to a new 96-well qPCR plate.

7.3 Transfer **1 µl of each Bisulfite-converted Library Pool** to the 96-well qPCR plate. Leave the rest of the samples at 4°C during the quantification by qPCR.

7.4 Seal the 96-well plate, mix by vortexing, centrifuge briefly and place it in a thermocycler and run the following program for 1 h.

Step	Temperature	Time	Cycles
Initial denaturation	95°C	5 min	1
Denaturation	98°C	20 sec	30
Annealing	60°C	15 sec	
Extension	72°C	45 sec	

7.5 Analyze the Ct values. Determine the number of cycles (n) needed for the library amplification. Typically n = Ct rounded to the nearest whole number, but this might need some optimization depending on your setup. E.g. if Ct= 6.89, apply 7 amplification cycles to your library; if Ct= 9.28, apply 9 amplification cycles to your library.

LIBRARY AMPLIFICATION

7.6 Prepare the **Amplification Mix** in a 1.5 ml nuclease-free tube as described in the following table.

		Mix for # samples (µl)			
Components	Volume/ reaction	2	4	8	16
MethylTaq Plus 2X Master Mix	25 µl	62.5	112.5	212.5	412.5
Primer Mix	2.5 µl	6.25	11.25	21.25	41.25
Nuclease-free Water	3.5 µl	8.75	15.75	29.75	57.75
Total volume	**31 µl**	**77.5**	**139.5**	**263.5**	**511.5**

7.7 Transfer **31 µl of Amplification Mix** to a new 96-well qPCR plate.

7.8 Transfer **19 µl of each Bisulfite-converted Library Pool** to the 96-well qPCR plate and mix by pipetting.

NOTE: *If you have to apply different number of amplification cycles to your libraries, prefer to distribute them in individual 0.2ml PCR tubes or strips rather than in 96-well plate.*

7.9 Place the 96-well PCR plate (or 0.2ml tube or strips) in a thermocycler with heated lid on (105°C) and run the following program.

Step	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	98°C	20 sec	Ct = n
Annealing	60°C	15 sec	
Extension	72°C	45 sec	
Final extension	72°C	7 min	1
Hold	4°C	∞	

NOTE: *The Ct value is highly dependent on the qPCR machine you use, as well as the way you analyze the qPCR results. Thus, when using the kit for the first time you may need to verify that the Ct = n rule can be applied in your conditions. As general guidelines, for a RRBS experiment starting from 100 ng of good quality DNA and with pools of 5 samples, the number of cycles to apply is typically between 7 and 10, depending on the pool.*

CAUTION: *We recommend not using more than 15 cycles in order to avoid any over-amplification.*

CLEAN-UP

CAUTION: *When processing pools from DNA starting amounts ≥ 50ng, proceed to 1 round of beads clean-up. When processing pools from DNA starting amounts < 50ng, proceed to 2 rounds of beads clean-up while using 1X beads ratio for the second round (50 µl library pool + 50* µl *beads).*

7.10 Carefully resuspend the **AMPure XP Beads** at room temperature and add **72.5 µl of beads** to each library pool in a 96-well plate. Mix by pipetting 10 times until the mixture is homogeneous.

7.11 Incubate at room temperature for at least 15 min.

7.12 Place the 96-well plate on a 96-well magnetic rack and wait until the beads are completely bound to the magnet (~5 min).

7.13 Carefully remove and discard the supernatant without disturbing the beads that contain the desired DNA fragments.

7.14 Wash the beads pellets twice as follows:

- With the 96-well plate on the 96-well magnetic rack, add **100 µl of freshly prepared 80% Ethanol** without disturbing the beads pellets and wait for 5 sec.

  ***CAUTION**: Do not leave the beads pellets longer than 5 sec in 80% Ethanol as it may already elute DNA from the beads.*

- Carefully remove and discard the supernatant without disturbing the beads pellets.
- Be sure to remove all visible liquid after the second wash.

7.15 Allow the beads pellets to air-dry at room temperature for 10 min.

7.16 Add **15 µl of Resuspension Buffer** to each library pool of the 96-well, vortex gently to resuspend the beads and spin down briefly.

7.17 Incubate at room temperature for 5 min out of the magnetic rack to elute DNA.

7.18 Place the 96-well plate on a 96-well magnetic rack and wait until the beads are completely bound to the magnet (~5 min).

7.19 Without disturbing the beads pellets, carefully aspirate and transfer the supernatants to a new 1.5 ml nuclease-free tube for each pool

STEP 8

Quality control & Next-Generation Sequencing

8.1 Determine the concentrations of your samples by the use of a fluorescence-based assay such as the **Qubit High Sensitivity assay (ThermoFischer Scientific)**.

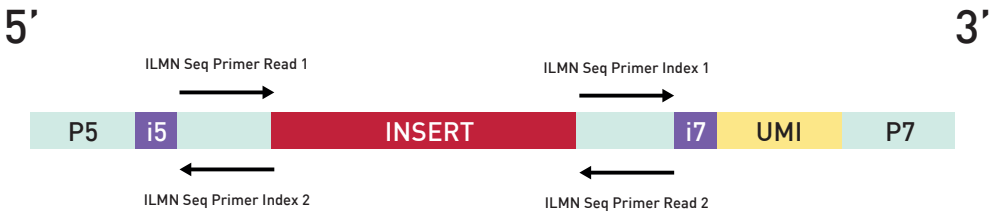
8.2 Run a part of each library on a **High Sensitivity chip for BioAnalyzer (Agilent)** or on **Fragment Analyzer (Agilent)** according to the manufacturer's instructions.

NOTE: *In some cases, for example when adapter dimers (150 bp peak) are still present, an additional clean-up can be performed. To do that, add nuclease-free water to the pool to reach 50 µL and repeat steps 7.10 to 7.18 but using 50 µl of beads according to the following table.*

		Additional clean-up (step 8)	
DNA starting amount	Initial clean-up (step 7)	Adapter dimer peak >5%	Adapter dimer peak 1-5%
100 – 50 ng	1 round	2 rounds	1 round
< 50 – 25 ng	2 rounds	2 rounds	1 round

Premium RRBS V2 Construct

Figure 1. Premium RRBS V2 library construct scheme. The final construct bears unique dual indexes (UDI) and an unique molecular identifier (UMI).



P5/P7 = Illumina adapters

UMI = Unique Molecular Identifier

i5/i7 = Unique Dual Indexes

Sequencing Recommendations

Paired-end sequencing with 50 bp read length is recommended because it is the most cost-effective solution, but it is also possible to use longer reads.

RRBS-UDI library construct contains the UMI sequence just behind the i7 index. Thus, to allow UMI sequence reading during sequencing, it is necessary to specify a particular run mode/recipe to your sequencing provider. The appropriate run mode is: **cycle 50-8-(i5) and 17-(i7)-50**. As a consequence, you should ask for delivery of fastq files with undetermined reads and perform the indexes and UMI processing yourself following our data analysis pipeline recommendations and tools (see section Data Analysis Recommendations, p37).

Due to the enzymatic digestion, all RRBS reads are starting by the same bases, which can reduce the efficiency of the base calling during the sequencing run. Moreover, bisulfite conversion of unmethylated C to T reduces library complexity. In order to overcome these difficulties, sequencing parameters for low diversity libraries should be applied during the sequencing of RRBS libraries. Hence, we recommend discussing this issue with your sequencing provider. For sequencing on NovaSeq platforms, we recommend using 15% PhiX spike-in.

Table 3. Recommended sequencing parameters for RRBS libraries for various Illumina's platforms

Step	MiSeq	HiSeq2000/2500	HiSeq3000/4000	NextSeq500/550	NovaSeq6000
Percentage of Illumina PhiX control	5%	5%	5%	20%	15%
Cluster density	Aim at a 30% beneath the optimal range for the chemistry version and platform used				
Softwatre version	RTA 1.17.28 or newer	HCS 2.2.38 or newer	HCS 3.4.0.38 or newer	NCS 1.3 or newer	NSCS 1.6 or newer

RRBS Data Analysis Recommendation

Important notice: the below recommendations are applicable for the version 2 of the kit only (Premium RRBS kit v2, Cat. No. C02030036 and C02030037) and not applicable for the version 1 of the kit.

Diagenode's RRBS V2 data sets display some particular features due to the technology and library construct used:

a) RRBS V2 libraries bear both a unique dual index and unique molecular identifier and require a specific data treatment to perform demultiplexing.

b) During RRBS workflow, genomic DNA is fragmented by using a restriction enzyme, MspI. Thus, all fragments are bearing a specific sequence corresponding to the MspI restriction site. Moreover, depending on the sequencing read length and library insert length, the reads might hold a portion of the adaptors. Thus, specific trimming is needed.

c) As all bisulfite converted libraries, RRBS V2 data sets need a dedicated tool for reads alignment.

d) RRBS V2 libraries are stranded. However, the insert strandness is inverted compared to the classical Illumina TruSeq orientation. This specific orientation of the reads has to be taken into account either before processing or during the alignment step.

e) RRBS libraries display a lower diversity than whole genome data sets due to their specific treatment inducing relatively short fragment size. This induces the possibility of PCR duplicates. To tackle this potential issue, RRBS V2 construct bear a 9bp Unique Molecular Identifiers (UMI) sequence used to distinguish real biological copies from PCR artefacts.

As a consequence of these particularities, the following specific data analysis tools are recommended:

Samples demultiplexing

To preserve the UMI information, a 2-step process is required for the demultiplexing of the sequencing data:

1. Use bcl2fastq to generate Undetermined fastq files from bcl files, keeping the UDI and UMI information.

2. Use fumi_tools to demultiplex the Undetermined fastq based on UDI sequences and append the UMI information at the end of the read names.

I. bcl2fastq

Following the completion of the sequencing run, the bcl2fastq converter tool must be run with default settings. An example is shown below:

```
bcl2fastq --runfolder-dir ./ --sample-sheet <bcl2fastq_sample_sheet.csv>
--output-dir ./Data/ \
 --ignore-missing-bcls \
 --ignore-missing-filter \
 --ignore-missing-positions \
 --ignore-missing-controls \
 --auto-set-to-zero-barcode-mismatches \
 --find-adapters-with-sliding-window \
 --adapter-stringency 0.9 \
 --loading-threads 4 \
 --processing-threads 10 \
 --writing-threads 4 \
 --mask-short-adapter-reads 22 \
 --minimum-trimmed-read-length 35
```

The sample sheet must not specify any UDI sequences at this step and should be formatted as follow:

```
[Header]
IEMFileVersion,5
Date,xx-xx-xx
WorkFlow,Generate FASTQ
Application, FASTQ Only
Instrument Type,NovaSeq6000
Assay,
Index Adapters,
Description,
Chemistry,
[Reads]
50
50
[Settings]
Adapter,
AdapterRead2,
[Data]
Sample_ID,Sample_Name,Sample_Plate,Sample_Well,Index_Plate_Well,I7_Index_ID,index,I5_Index_ID,index2,Sample_Project
RRBS_V2_samples,,,,,,NNNNNNNNNNNNNNNN,,NNNNNNNN,RRBS_V2_Project
```

Note: *Individual lanes can be selected using the --tiles s_x parameter in the bcl2fastq command line, where x represents the lane to be processed. Alternatively, the lane can be specified in the sample sheet as below:*

```
Lane,Sample_ID,Sample_Name,Sample_Plate,Sample_Well,Index_Plate_Well,I7_Index_ID,index,I5_Index_ID,index2,Sample_Project
x,RRBS_V2_samples,,,,,,NNNNNNNNNNNNNNNN,,NNNNNNNN,RRBS_V2_Project
```

Using this sample sheet, the bcl2fastq command generates one pair of Undetermined fastq files per processed lane (Undetermined_S0_L00x_R1_001.fastq.gz and Undetermined_S0_L00x_R2_001.fastq.gz), which are formatted with the <index7><UMI>+<index5> appended at the end of the read names:

```
@A00801:392:HGNKCDRX3:1:1101:1036:1000 1:N:0:CTCTCGTCCCTATCCAC+AGGTTATA
NAAAAACCTTCCCAACATAAAACTTAATAATAACTTTAAACAACACCCATC
+
#F,:FFFFFFFFFFFFF:FFFFFFFFFFFFFFFFFFFF:FFFFFFFFFF
```

II. fumi_tools

The Undetermined fastq files can subsequently be used with the demultiplex function of fumi_tools using the following command (more information can be found on the gitlab page of the tool: https://ccb-gitlab.cs.uni-saarland.de/tobias/fumi_tools) :

```
fumi_tools demultiplex -i Undetermined_S0_L00x_R1_001.fastq.gz \
--input-read2 Undetermined_S0_L00x_R2_001.fastq.gz \
 -s <demultiplex_sample_sheet.csv> \
-o ./pathToDemultiplexLibrary /%s_S%i_L%l_R%r_001. fastq.gz \
--threads 6 --format-umi --tag-umi \
```

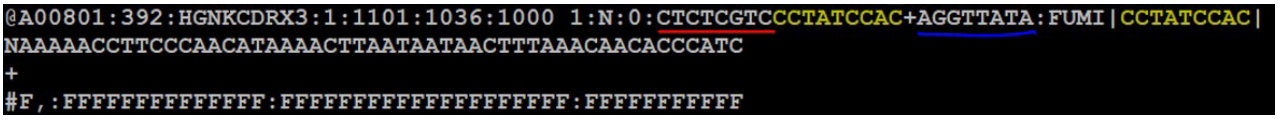
The sample sheet used with fumi_tools must list the samples and the corresponding sequences of the UDI used:

```
Sample_ID,Sample_Name,Lane,index2,index
1, RRBS_V2_sample1,1,AGGTTATA,CTCTCGTC
2, RRBS_V2_sample2,1,GGTCACGA,GGCTTAAG
3, RRBS_V2_sample3,2,GGTCACGA,GGCTTAAG
4, RRBS_V2_sample4,2,AACTGTAG,AATCCGGA
```

Where the index2 column corresponds to the sequence of the i7 index and the index column corresponds to the sequence or the reverse complement of the i5 index depending on the type of sequencer used (see Tables 3a. and 3b.). The lane onto which the sample was loaded can be specified.

The fumi_tools demultiplex generates one pair of fastq files per sample, assigned based on the UDI sequences, in which the UMI sequences have been extracted and appended at the end of the read names as follow:

<index7><UMI>+<index5>:FUMI|<UMI>|



Read trimming

Any software for reads trimming, like cutAdapt or Trimmomatic, can be used. However, Diagenode recommends using Trim Galore! for reads trimming as this tool has specific parameters to accurately trim MspI-generated RRBS libraries and remove artefactual bases at the end of reads. The following command can be used to trim read pairs with Trim_Galore:

trim_galore -j <number of cores> --rrbs --non_directional --paired --length 15 --fastqc R1.fq.gz R2.fq.gz -o <output_directory>

where:

- R1.fastq.gz, R2.fastq.gz: are the paths to the files of R1 and R2 fastq files from the sequencing.
- -rrbs and --non_directional: specifies that the inputs are MspI digested libraries and trim the cytosine positions that were filled-in during the end-repair step to avoid using these cytosines for the methyaltion calls
- --paired: specifies quality and adaptor trimming for paired-end libraires
- --length 15: is the minimal length of the reads kept after trimming
- --fastqc: performs a FastQC on the read pairs after trimming

Read alignment

- In RRBS data sets, as DNA fragments underwent bisulfite conversion, the reads will not mirror the genomic sequence accurately. Indeed, based on the cytosine status (methylated-

unconverted or unmethylated-converted) there could be different versions of the same read. Therefore, a conventional NGS read aligner like BWA or Bowtie cannot handle bisulfite converted datasets. A dedicated BS aligner, such as Bismark or BSMAP (with specific version for RRBS, RRBSMAP), should be used.

- The RRBS V2 libraries are inverted compared to classical Illumina orientation and needs to be aligned with Bismark using the "--pbat" option. The pbat option will essentially do the exact opposite of an alignment in directional mode by aligning the read pairs to the CTOT (complementary to original top) and CTOB (complementary to original bottom) strands instead of OT (original top) and OB (original bottom). If using any other aligner, please refer to its dedicated documentation for such settings.

PCR deduplication

The sequence of the UMI can be used in order to remove read pairs aligning at the same genomic coordinates of the reference genome and that carry the same UMI. To carry out this step the following set of commands can be used:

- Sort the aligned bam file by genomic coordinates:
  - *samtools sort aligned_rrbs.bam -o sorted.bam*
  - Where:
    - aligned_rrbs.bam = the bam file generated by bismark or any other aligner. for rrbs.
    - sorted.bam = the output file with the sorted reads.

- Remove the duplicated read pairs using the following command line:
  - *o fumi_tools dedup -i sorted.bam -o dedup.bam --threads 6 --memory 3G --paired*
  - Where:
    - sorted.bam = the coordinates-sorted bam generated by samtools.
    - dedup.bam = the output bam file with the deduplicated reads generated by fumi_tools

DNA methylation extraction

Once the reads are aligned, the methylation levels of the bases can be extracted. Many aligners, like the aforementioned Bismark and RRBSMAP come with a built-in methylation extractor module that produces this output, along with general methylation statistics.

From the output it is easy to proceed with the desired downstream analysis. For example, bedGraph or WIG files can be created which can be viewed in a browser like the UCSC Genome Browser to check the methylation levels visually. Interesting regions can be extracted into BED files based on methylation criteria, and be annotated to see which genes are affected by hyper- or hypo-methylation.

Spike-in controls

Diagenode's RRBS Kit V2 contains methylated and unmethylated spike-in controls to monitor the efficiency of bisulfite conversion. Because their methylation status is known, they can be used to assess the conversion rate.

Please download our Premium RRBS kit v2 Spike-in controls analysis to estimate bisulfite conversion efficiency manual to know how to optimally use our spike-in controls. You can find this document on RRBS v2 product page.

Links for useful tools:

Tool	Link
Bismark	http://www.bioinformatics.babraham.ac.uk/projects/bismark
BSMAP	https://code.google.com/p/bsmap
RRBSMAP	http://omictools.com/rrbsmap-s996.html
Trim Galore!	http://www.bioinformatics.babraham.ac.uk/projects/trim_galore
Trimmomatic	http://www.usadellab.org/cms/index.php?page=trimmomatic
UCSC Genome Browser	http://genome.ucsc.edu/cgi-bin/hgGateway
Fumi tools	https://ccb-gitlab.cs.uni-saarland.de/tobias/fumi_tools
bcl2fastq	https://emea.support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-software.html

Example of Results

Figure 2: BioAnalyzer (Agilent) High Sensitivity DNA chip profiles of Premium RRBS V2 libraries prepared from 100 ng (A) and 50 ng (B) human gDNA (pooled by 5).

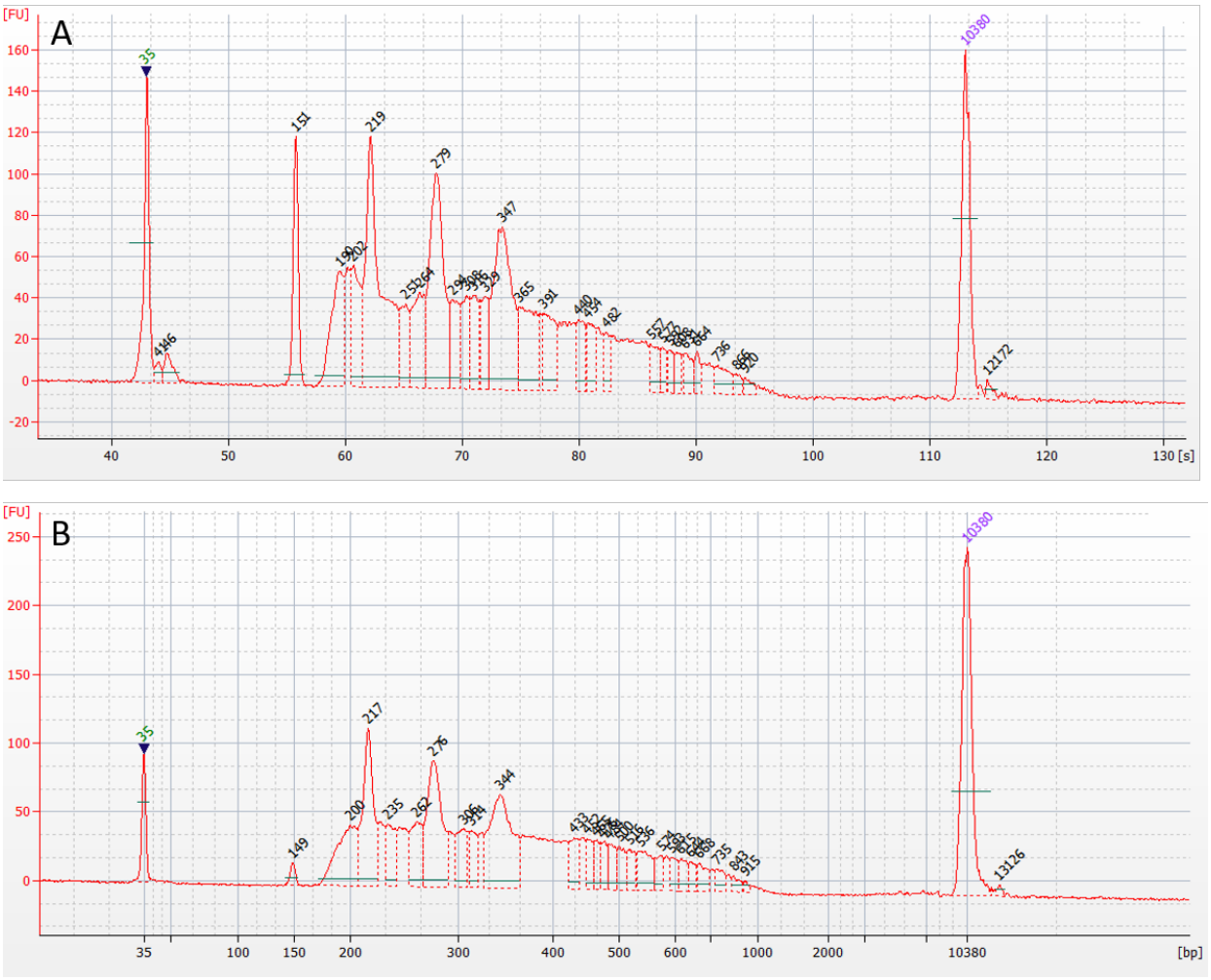


Figure 3: BioAnalyzer (Agilent) High Sensitivity DNA chip profiles of Premium RRBS V2 libraries prepared from 25 ng human gDNA (pooled by 5) (A) before additional clean-up and (B) after two additional clean-up to remove remaining adapter-dimer peak.

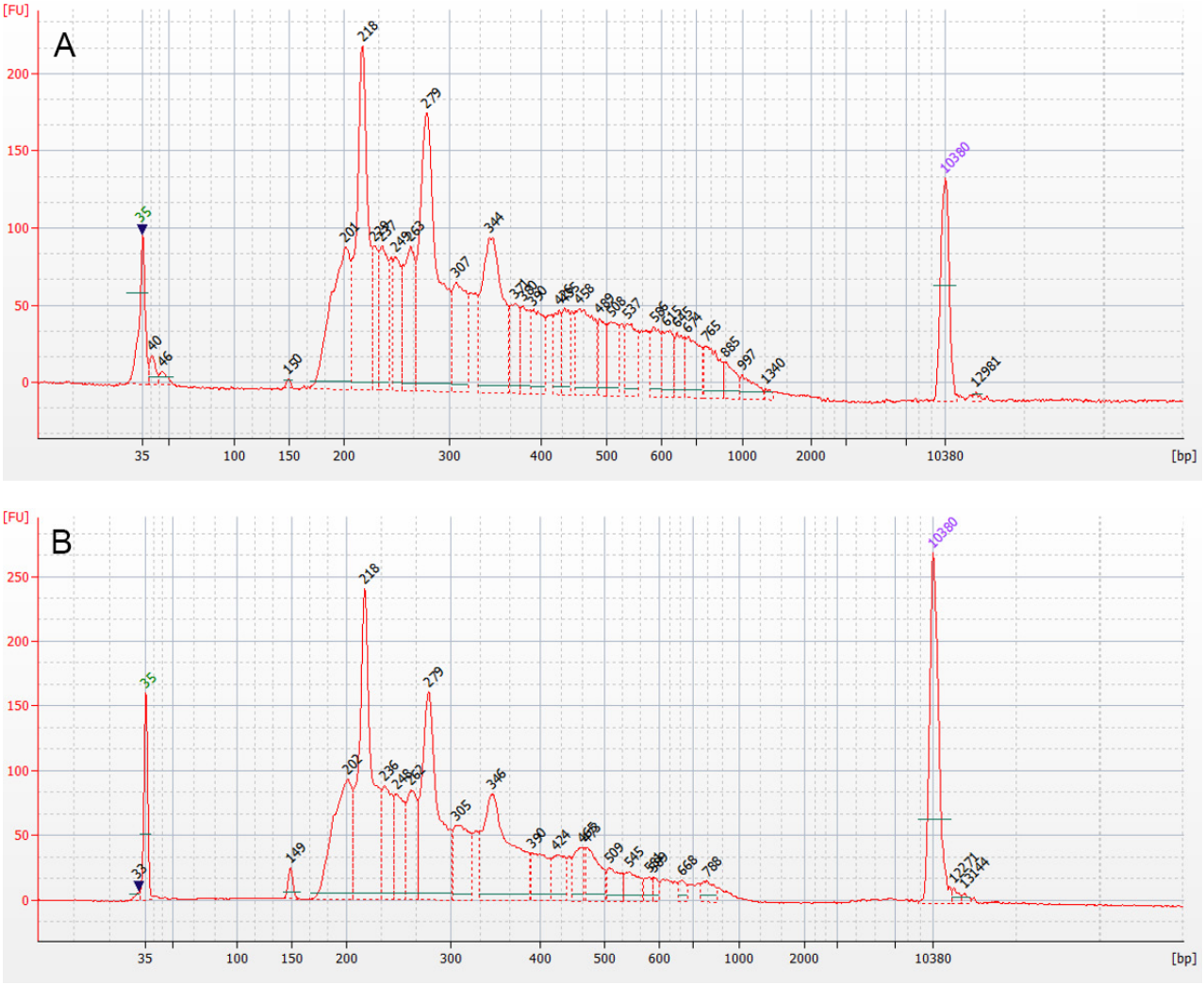


Table 4: Examples of Premium RRBS V2 sequencing data. Premium RRBS V2 libraries were prepared from different starting amounts of human gDNA pooled by 5 samples and sequenced in paired-end 50 bp on Illumina NovaSeq 6000 instrument generating 30-40 million read pairs per sample. UMI data processing enables accurate estimation of CpG counts.

DNA starting amount	Before UMI processing and PCR duplicates removal			After UMI processing and PCR duplicates removal		
	No. of CpGs detected	No. of CpGs detected with coverage >10x	Average CpG coverage	No. of CpGs detected	No. of CpGs detected with coverage >10x	Average CpG coverage
100 ng	7.6 M	4.4 M	14	7.6 M	3.7 M	11
50 ng	7.4 M	4.3 M	13	7.4 M	2.7 M	9
25 ng	7.4 M	3.8 M	13	7.4 M	1.9 M	7

Additional Protocol - Manual Calculation of Volumes for Pooling

M.1 Copy the following informations concerning your samples in a new Excel file:

- Column A - Position in the 96-well plate (A01 to H12)
- Column B - Sample name
- Column C - Indexed-adapter number (1, 2, 3...)
- Column D - Ct values from your qPCR analysis software (replicate 1)
- Column E - Ct values from your qPCR analysis software (replicate 2)

M.2 Calculate the average Ct value from the qPCR duplicates in column F.

M.3 Identify projects that should be sequenced separately (e.g. samples from different species) and insert an empty row between each project.

Figure 4: Screenshot of the Sample Sheet containing sorted samples, barcodes, Ct and average Ct values.

	A	B	C	D	E	F
1	**Well**	**Sample name**	**Adaptor ID**	**Ct 1**	**Ct 2**	**Average Ct**
2	A01	Sample 1	17	4.46	4.6	4.53
3	A02	Sample 2	24	4.52	4.51	4.515
4	A03	Sample 3	26	4.29	3.99	4.14
5	A04	Sample 4	28	3.92	4.13	4.025
6	A05	Sample 5	29	4.24	3.93	4.085
7	A06	Sample 6	30	3.77	3.82	3.795
8	A07	Sample 7	31	4.23	4.24	4.235
9	A08	Sample 8	32	4.31	4.44	4.375
10	A09	Sample 9	33	4.46	4.51	4.485
11	A10	Sample 10	34	4.38	4.32	4.35
12	A11	Sample 11	35	4.33	4.47	4.4
13	A12	Sample 12	36	4.34	4.4	4.37
14	B01	Sample 13	37	4.58	4.6	4.59
15	B02	Sample 14	38	4.85	4.45	4.65
16	B03	Sample 15	39	4.14	4.54	4.34
17	B04	Sample 16	40	3.94	4.17	4.055
18	B05	Sample 17	41	4.21	4.63	4.42
19	B06	Sample 18	42	4.44	4.42	4.43

M.4 For each project:

- Select all rows belonging to the project and sort them by column F (average Ct value) in descending order.

NOTE: *This ensures that samples with similar Ct values are pooled together.*

- Decide on pools of n samples and color their rows accordingly. In the same pool, samples must have similar Ct values, similar features (species, FFPE or not...) and different adapters ID.

NOTE: *If the project sample number is not a multiple of the decided multiplexing level (e.g. 10), we recommend using the samples with the smallest Ct values for a larger pool and the samples with the highest Ct values for a smaller pool.*

M.5 For each pool, identify adapter barcodes clashes by selecting the adapter IDs in column C and applying **Conditional Formatting › Highlight Cells Rules › Duplicate Values.**

- Resolve adapter clashes by exchanging rows via cut and paste. Ct values should be as similar as possible.

- Sort each pool by column F in descending order (swapping rows might have messed up the order).

Figure 5: Screenshot of the Sample Sheet after clashed barcode identification (up) and column sorting (down).

A	B	C	D	E	F
Well	**Sample name**	**Adaptor ID**	**Ct 1**	**Ct 2**	**Average Ct**
B02	Sample 14	38	4.85	4.45	4.65
B01	Sample 13	37	4.58	4.6	4.59
A01	Sample 1	17	4.46	4.6	4.53
A02	Sample 2	24	4.52	4.51	4.515
B07	Sample 19	43	4.39	4.61	4.5
A09	Sample 9	33	4.46	4.51	4.485
C01	Sample 25	17	4.4	4.53	4.465
C03	Sample 27	26	4.6	4.28	4.44
B06	Sample 18	42	4.44	4.42	4.43
B05	Sample 17	41	4.21	4.63	4.42
A11	Sample 11	35	4.33	4.47	4.4
B08	Sample 20	44	4.31	4.46	4.385
A08	Sample 8	32	4.31	4.44	4.375

A	B	C	D	E	F
Well	**Sample name**	**Adaptor ID**	**Ct 1**	**Ct 2**	**Average Ct**
B02	Sample 14	38	4.85	4.45	4.65
B01	Sample 13	37	4.58	4.6	4.59
A01	Sample 1	17	4.46	4.6	4.53
A02	Sample 2	24	4.52	4.51	4.515
B07	Sample 19	43	4.39	4.61	4.5
A09	Sample 9	33	4.46	4.51	4.485
C03	Sample 27	26	4.6	4.28	4.44
C01	Sample 25	17	4.4	4.53	4.465
B06	Sample 18	42	4.44	4.42	4.43
B05	Sample 17	41	4.21	4.63	4.42
A11	Sample 11	35	4.33	4.47	4.4
B08	Sample 20	44	4.31	4.46	4.385
A08	Sample 8	32	4.31	4.44	4.375

M.6 In column G, calculate the difference in Ct values (dCt) for each sample as: highest average Ct in pool – average Ct of the sample **(Ctmax-Ctsample)**. Use $ signs to fix the Ctmax in the formula inside each pool.

M.7 Calculate the pooling volume in column H as **17*2^(-dCt)**.

NOTE: *In each pool, 17 µl of the sample with the Ctmax will be used.*

M.8 In column I, calculate the volume of water to add to each pool to reach 120 µl with the following formula: **water volume = 120 - (sum each pool volume)** where "pool vol" is the pooling volume of each sample.

> ***NOTE:*** *If the number of samples per pool is high, the volume of water calculated may be negative and the total volume of the pool may be higher than 120 µl which is not a problem. If this is the case, do not add water and calculate the total volume of the pool, as you will need it to calculate the amount of DNA Binding buffer to be added. The pool will have to be loaded in two steps on the DNA Spin Column at step 5.5 as described in the notes.*

M.9 In column J, specify pool's name in which you will combine the samples.

Figure 6: Screenshot of the Sample Sheet after clashed barcode identification (left) and column sorting (right).

	A	B	C	D	E	F	G	H	I	J
1	**Well**	**Sample name**	**Adaptor ID**	**Ct 1**	**Ct 2**	**Average Ct**	**dCt**	**Pooling volume**	**Water volume**	**Pool name**
2	B02	Sample 14	38	4.85	4.45	4.65	0	17.0	10.4	Pool 1
3	B01	Sample 13	37	4.58	4.6	4.59	0,06	16.3		
4	A01	Sample 1	17	4.46	4.6	4.53	0,12	15.6		
5	A02	Sample 2	24	4.52	4.51	4.515	0,135	15.5		
6	B07	Sample 19	43	4.39	4.61	4.5	0,15	15.3		
7	A09	Sample 9	33	4.46	4.51	4.485	0,165	15.2		
8	C03	Sample 27	26	4.6	4.28	4.44	0,21	14.7		
9	C01	Sample 25	17	4.4	4.53	4.465	0	17	5.7	Pool 2
10	B06	Sample 18	42	4.44	4.42	4.43	0,035	16.6		
11	B05	Sample 17	41	4.21	4.63	4.42	0,045	16.5		
12	A11	Sample 11	35	4.33	4.47	4.4	0,065	16.2		
13	B08	Sample 20	44	4.31	4.46	4.385	0,08	16.1		
14	A08	Sample 8	32	4.31	4.44	4.375	0,09	16.0		
15	A12	Sample 12	36	4.34	4.4	4.37	0,095	15.9		
16	A10	Sample 10	34	4.38	4.32	4.35	0	17	13.2	Pool 3
17	B03	Sample 15	39	4.14	4.54	4.34	0,01	16.9		
18	A07	Sample 7	31	4.23	4.24	4.235	0,115	15.7		
19	A03	Sample 3	26	4.29	3.99	4.14	0,21	14.7		
20	C04	Sample 28	28	4	4.24	4.12	0,23	14.5		
21	A05	Sample 5	29	4.24	3.93	4.085	0,265	14.1		
22	B04	Sample 16	40	3.94	4.17	4.055	0,295	13.9		
23	A04	Sample 4	28	3.92	4.13	4.025	0	17	32.4	Pool 4
24	B09	Sample 21	45	3.8	4.01	3.905	0,12	15.6		
25	A06	Sample 6	30	3.77	3.82	3.795	0,23	14.5		
26	B11	Sample 23	47	3.26	3.51	3.385	0,64	10.9		
27	B12	Sample 24	48	3.21	3.54	3.375	0,65	10.8		
28	C02	Sample 26	24	2.97	3.52	3.245	0,78	9.9		
29	B10	Sample 22	46	2.67	3.47	3.07	0,955	8.8		
30										
31	C11	Sample 35	35	4.18	4.89	4.535	0	17	34.9	Pool 5
32	C09	Sample 33	33	4.02	4.53	4.275	0,26	14.2		
33	C06	Sample 30	30	3.67	4.36	4.015	0,52	11.8		
34	C12	Sample 36	36	3.9	4	3.95	0,585	11.3		
35	C10	Sample 34	34	3.16	3.91	3.535	1	8.5		
36	C07	Sample 31	31	3.41	3.64	3.525	1,01	8.4		
37	C08	Sample 32	32	3.13	3.34	3.235	1,3	6.9		
38	C05	Sample 29	29	3.06	3.38	3.22	1,315	6.8		

M.10 Go back to step 5.1 and follow the protocol.

Premium Methyl UDI-UMI Adapters Sequences

Specific Premium Methyl UDI-UMI Adapters were designed with UDI and UMI and validated for Premium RRBS experiments. These adapters are not included in this kit and should be ordered separately to proceed with RRBS library preparation. All Premium Methyl UDI-UMI Adapters are supplied at 100 nM in a volume sufficient for 1 rxn (5 µl).

Table 5a. Premium Methyl UDI-UMI Adapters Sequences– Set A (1-24)

Premium Methyl UDI-UMI Adapter #	i5 Index NovaSeq 6000 v1.0, MiSeq, HiSeq 2000/2500 systems	i5 index NovaSeq 6000 v1.5, iSeq, MiniSeq, NextSeq, HiSeq 3000/4000 systems	i7 index* (all Illumina systems)
1	AGCGCTAG	CTAGCGCT	CCGCGGTTNNNNNNNNN
2	GATATCGA	TCGATATC	TTATAACCNNNNNNNNN
3	CGCAGACG	CGTCTGCG	GGACTTGGNNNNNNNNN
4	TATGAGTA	TACTCATA	AAGTCCAANNNNNNNNN
5	AGGTGCGT	ACGCACCT	ATCCACTGNNNNNNNNN
6	GAACATAC	GTATGTTC	GCTTGTCANNNNNNNNN
7	ACATAGCG	CGCTATGT	CAAGCTAGNNNNNNNNN
8	GTGCGATA	TATCGCAC	TGGATCGANNNNNNNNN
9	CCAACAGA	TCTGTTGG	AGTTCAGGNNNNNNNNN
10	TTGGTGAG	CTCACCAA	GACCTGAANNNNNNNNN
11	CGCGGTTC	GAACCGCG	TCTCTACTNNNNNNNNN
12	TATAACCT	AGGTTATA	CTCTCGTCNNNNNNNNN
13	AAGGATGA	TCATCCTT	CCAAGTCTNNNNNNNNN
14	GGAAGCAG	CTGCTTCC	TTGGACTCNNNNNNNNN
15	TCGTGACC	GGTCACGA	GGCTTAAGNNNNNNNNN
16	CTACAGTT	AACTGTAG	AATCCGGANNNNNNNNN
17	ATATTCAC	GTGAATAT	TAATACAGNNNNNNNNN
18	GCGCCTGT	ACAGGCGC	CGGCGTGANNNNNNNNN
19	ACTCTATG	CATAGAGT	ATGTAAGTNNNNNNNNN
20	GTCTCGCA	TGCGAGAC	GCACGGACNNNNNNNNN
21	AAGACGTC	GACGTCTT	GGTACCTTNNNNNNNNN

Premium Methyl UDI-UMI Adapter #	i5 Index NovaSeq 6000 v1.0, MiSeq, HiSeq 2000/2500 systems	i5 index NovaSeq 6000 v1.5, iSeq, MiniSeq, NextSeq, HiSeq 3000/4000 systems	i7 index* (all Illumina systems)
22	GGAGTACT	AGTACTCC	AACGTTCCNNNNNNNNN
23	ACCGGCCA	TGGCCGGT	GCAGAATTNNNNNNNNN
24	GTTAATTG	CAATTAAC	ATGAGGCCNNNNNNNNN

**In i7 index sequence, the 9N indicates the UMI sequence.*

Table 5b. Premium Methyl UDI-UMI Adpaters Sequences – Set B (25-48)

Premium Methyl UDI-UMI Adapter #	i5 Index NovaSeq 6000 v1.0, MiSeq, HiSeq 2000/2500 systems	i5 index NovaSeq 6000 v1.5, iSeq, MiniSeq, NextSeq, HiSeq 3000/4000 systems	i7 index* (all Illumina systems)
25	AACCGCGG	CCGCGGTT	ACTAAGATNNNNNNNNN
26	GGTTATAA	TTATAACC	GTCGGAGCNNNNNNNNN
27	CCAAGTCC	GGACTTGG	CTTGGTATNNNNNNNNN
28	TTGGACTT	AAGTCCAA	TCCAACGCNNNNNNNNN
29	CAGTGGAT	ATCCACTG	CCGTGAAGNNNNNNNNN
30	TGACAAGC	GCTTGTCA	TTACAGGANNNNNNNNN
31	CTAGCTTG	CAAGCTAG	GGCATTCTNNNNNNNNN
32	TCGATCCA	TGGATCGA	AATGCCTCNNNNNNNNN
33	CCTGAACT	AGTTCAGG	TACCGAGGNNNNNNNNN
34	TTCAGGTC	GACCTGAA	CGTTAGAANNNNNNNNN
35	AGTAGAGA	TCTCTACT	AGCCTCATNNNNNNNNN
36	GACGAGAG	CTCTCGTC	GATTCTGCNNNNNNNNN
37	AGACTTGG	CCAAGTCT	TCGTAGTGNNNNNNNNN
38	GAGTCCAA	TTGGACTC	CTACGACANNNNNNNNN
39	CTTAAGCC	GGCTTAAG	TAAGTGGTNNNNNNNNN
40	TCCGGATT	AATCCGGA	CGGACAACNNNNNNNNN
41	CTGTATTA	TAATACAG	ATATGGATNNNNNNNNN
42	TCACGCCG	CGGCGTGA	GCGCAAGCNNNNNNNNN
43	ACTTACAT	ATGTAAGT	AAGATACTNNNNNNNNN
44	GTCCGTGC	GCACGGAC	GGAGCGTCNNNNNNNNN
45	AAGGTACC	GGTACCTT	ATGGCATGNNNNNNNNN
46	GGAACGTT	AACGTTCC	GCAATGCANNNNNNNNN
47	AATTCTGC	GCAGAATT	GTTCCAATNNNNNNNNN
48	GGCCTCAT	ATGAGGCC	ACCTTGGCNNNNNNNNN

**In i7 index sequence, the 9N indicates the UMI sequence.*

Table 5c. Premium Methyl UDI-UMI Adapters Sequences - 96 rxns (1-96)

Premium Methyl UDI-UMI Adapter #	Well position	i5 Index (HiSeq® 2000/2500, MiSeq®, NovaSeq® systems)	i5 index (HiSeq 3000/4000/X, NextSeq®, MiniSeq®, iSeq® systems)	i7 index* (all Illumina systems)
1	A01	AGCGCTAG	CTAGCGCT	CCGCGGTTNNNNNNNNN
2	A02	GATATCGA	TCGATATC	TTATAACCNNNNNNNNN
3	A03	CGCAGACG	CGTCTGCG	GGACTTGGNNNNNNNNN
4	A04	TATGAGTA	TACTCATA	AAGTCCAANNNNNNNNN
5	A05	AGGTGCGT	ACGCACCT	ATCCACTGNNNNNNNNN
6	A06	GAACATAC	GTATGTTC	GCTTGTCANNNNNNNNN
7	A07	ACATAGCG	CGCTATGT	CAAGCTAGNNNNNNNNN
8	A08	GTGCGATA	TATCGCAC	TGGATCGANNNNNNNNN
9	A09	CCAACAGA	TCTGTTGG	AGTTCAGGNNNNNNNNN
10	A10	TTGGTGAG	CTCACCAA	GACCTGAANNNNNNNNN
11	A11	CGCGGTTC	GAACCGCG	TCTCTACTNNNNNNNNN
12	A12	TATAACCT	AGGTTATA	CTCTCGTCNNNNNNNNN
13	B01	AAGGATGA	TCATCCTT	CCAAGTCTNNNNNNNNN
14	B02	GGAAGCAG	CTGCTTCC	TTGGACTCNNNNNNNNN
15	B03	TCGTGACC	GGTCACGA	GGCTTAAGNNNNNNNNN
16	B04	CTACAGTT	AACTGTAG	AATCCGGANNNNNNNNN
17	B05	ATATTCAC	GTGAATAT	TAATACAGNNNNNNNNN
18	B06	GCGCCTGT	ACAGGCGC	CGGCGTGANNNNNNNNN
19	B07	ACTCTATG	CATAGAGT	ATGTAAGTNNNNNNNNN
20	B08	GTCTCGCA	TGCGAGAC	GCACGGACNNNNNNNNN
21	B09	AAGACGTC	GACGTCTT	GGTACCTTNNNNNNNNN
22	B10	GGAGTACT	AGTACTCC	AACGTTCCNNNNNNNNN
23	B11	ACCGGCCA	TGGCCGGT	GCAGAATTNNNNNNNNN
24	B12	GTTAATTG	CAATTAAC	ATGAGGCCNNNNNNNNN
25	C01	AACCGCGG	CCGCGGTT	ACTAAGATNNNNNNNNN
26	C02	GGTTATAA	TTATAACC	GTCGGAGCNNNNNNNNN
27	C03	CCAAGTCC	GGACTTGG	CTTGGTATNNNNNNNNN
28	C04	TTGGACTT	AAGTCCAA	TCCAACGCNNNNNNNNN
29	C05	CAGTGGAT	ATCCACTG	CCGTGAAGNNNNNNNNN
30	C06	TGACAAGC	GCTTGTCA	TTACAGGANNNNNNNNN
31	C07	CTAGCTTG	CAAGCTAG	GGCATTCTNNNNNNNNN
32	C08	TCGATCCA	TGGATCGA	AATGCCTCNNNNNNNNN

33	C09	CCTGAACT	AGTTCAGG	TACCGAGGNNNNNNNNN
34	C10	TTCAGGTC	GACCTGAA	CGTTAGAANNNNNNNNN
35	C11	AGTAGAGA	TCTCTACT	AGCCTCATNNNNNNNNN
36	C12	GACGAGAG	CTCTCGTC	GATTCTGCNNNNNNNNN
37	D01	AGACTTGG	CCAAGTCT	TCGTAGTGNNNNNNNNN
38	D02	GAGTCCAA	TTGGACTC	CTACGACANNNNNNNNN
39	D03	CTTAAGCC	GGCTTAAG	TAAGTGGTNNNNNNNNN
40	D04	TCCGGATT	AATCCGGA	CGGACAACNNNNNNNNN
41	D05	CTGTATTA	TAATACAG	ATATGGATNNNNNNNNN
42	D06	TCACGCCG	CGGCGTGA	GCGCAAGCNNNNNNNNN
43	D07	ACTTACAT	ATGTAAGT	AAGATACTNNNNNNNNN
44	D08	GTCCGTGC	GCACGGAC	GGAGCGTCNNNNNNNNN
45	D09	AAGGTACC	GGTACCTT	ATGGCATGNNNNNNNNN
46	D10	GGAACGTT	AACGTTCC	GCAATGCANNNNNNNNN
47	D11	AATTCTGC	GCAGAATT	GTTCCAATNNNNNNNNN
48	D12	GGCCTCAT	ATGAGGCC	ACCTTGGCNNNNNNNNN
49	E01	ATCTTAGT	ACTAAGAT	ATATCTCGNNNNNNNNN
50	E02	GCTCCGAC	GTCGGAGC	GCGCTCTANNNNNNNNN
51	E03	ATACCAAG	CTTGGTAT	AACAGGTTNNNNNNNNN
52	E04	GCGTTGGA	TCCAACGC	GGTGAACCNNNNNNNNN
53	E05	CTTCACGG	CCGTGAAG	CAACAATGNNNNNNNNN
54	E06	TCCTGTAA	TTACAGGA	TGGTGGCANNNNNNNNN
55	E07	AGAATGCC	GGCATTCT	AGGCAGAGNNNNNNNNN
56	E08	GAGGCATT	AATGCCTC	GAATGAGANNNNNNNNN
57	E09	CCTCGGTA	TACCGAGG	TGCGGCGTNNNNNNNNN
58	E10	TTCTAACG	CGTTAGAA	CATAATACNNNNNNNNN
59	E11	ATGAGGCT	AGCCTCAT	GATCTATCNNNNNNNNN
60	E12	GCAGAATC	GATTCTGC	AGCTCGCTNNNNNNNNN
61	F01	CACTACGA	TCGTAGTG	CGGAACTGNNNNNNNNN
62	F02	TGTCGTAG	CTACGACA	TAAGGTCANNNNNNNNN
63	F03	ACCACTTA	TAAGTGGT	TTGCCTAGNNNNNNNNN
64	F04	GTTGTCCG	CGGACAAC	CCATTCGANNNNNNNNN
65	F05	ATCCATAT	ATATGGAT	ACACTAAGNNNNNNNNN
66	F06	GCTTGCGC	GCGCAAGC	GTGTCGGANNNNNNNNN
67	F07	AGTATCTT	AAGATACT	TTCCTGTTNNNNNNNNN
68	F08	GACGCTCC	GGAGCGTC	CCTTCACCNNNNNNNNN
69	F09	CATGCCAT	ATGGCATG	GCCACAGGNNNNNNNNN
70	F10	TGCATTGC	GCAATGCA	ATTGTGAANNNNNNNNN

71	F11	ATTGGAAC	GTTCCAAT	ACTCGTGTNNNNNNNNN
72	F12	GCCAAGGT	ACCTTGGC	GTCTACACNNNNNNNNN
73	G01	CGAGATAT	ATATCTCG	CAATTAACNNNNNNNNN
74	G02	TAGAGCGC	GCGCTCTA	TGGCCGGTNNNNNNNNN
75	G03	AACCTGTT	AACAGGTT	AGTACTCCNNNNNNNNN
76	G04	GGTTCACC	GGTGAACC	GACGTCTTNNNNNNNNN
77	G05	CATTGTTG	CAACAATG	TGCGAGACNNNNNNNNN
78	G06	TGCCACCA	TGGTGGCA	CATAGAGTNNNNNNNNN
79	G07	CTCTGCCT	AGGCAGAG	ACAGGCGCNNNNNNNNN
80	G08	TCTCATTC	GAATGAGA	GTGAATATNNNNNNNNN
81	G09	ACGCCGCA	TGCGGCGT	AACTGTAGNNNNNNNNN
82	G10	GTATTATG	CATAATAC	GGTCACGANNNNNNNNN
83	G11	GATAGATC	GATCTATC	CTGCTTCCNNNNNNNNN
84	G12	AGCGAGCT	AGCTCGCT	TCATCCTTNNNNNNNNN
85	H01	CAGTTCCG	CGGAACTG	AGGTTATANNNNNNNNN
86	H02	TGACCTTA	TAAGGTCA	GAACCGCGNNNNNNNNN
87	H03	CTAGGCAA	TTGCCTAG	CTCACCAANNNNNNNNN
88	H04	TCGAATGG	CCATTCGA	TCTGTTGGNNNNNNNNN
89	H05	CTTAGTGT	ACACTAAG	TATCGCACNNNNNNNNN
90	H06	TCCGACAC	GTGTCGGA	CGCTATGTNNNNNNNNN
91	H07	AACAGGAA	TTCCTGTT	GTATGTTCNNNNNNNNN
92	H08	GGTGAAGG	CCTTCACC	ACGCACCTNNNNNNNNN
93	H09	CCTGTGGC	GCCACAGG	TACTCATANNNNNNNNN
94	H10	TTCACAAT	ATTGTGAA	CGTCTGCGNNNNNNNNN
95	H11	ACACGAGT	ACTCGTGT	TCGATATCNNNNNNNNN
96	H12	GTGTAGAC	GTCTACAC	CTAGCGCTNNNNNNNNN

FAQs

Can I use less than 100 ng DNA?

Our Premium RRBS Protocol has been optimized for 100 ng down to 25 ng of gDNA and gives very reproducible results for this amount. If the quality of the DNA is very good, RRBS could be performed with less DNA. However, we would not recommend using less than 10 ng.

Is the RRBS kit applicable to FFPE samples?

Yes, as soon as the DNA is not too much degraded (size>2000 bp). However, the number of CpGs covered is often reduced (from 0.7 to 2.5 M for human FFPE samples). We recommend increasing the starting amount of DNA (up to 400 ng) to balance the poor quality of the DNA.

Is the RRBS kit compatible with plant samples?

Technically, RRBS is compatible with any kind of genomic DNA, even if it has never been tested on plant DNA. The technique is based on an enrichment in CpG-rich regions (mainly CpG islands) which makes sense to study DNA methylation in vertebrates. In plants, the distribution of DNA methylation is quite different, with some 5-mC in various contexts other than CpG (around 45% CpG, 25% CHH, 30% CGH contexts), and therefore the relevance of standard RRBS protocol can be questioned.

Which DNA size is selected during the experiment?

The protocol selects fragments of 170 bp and above which corresponds to an insert size of 25 bp and above. The goal of our size selection is to catch the smaller fragments (corresponding to the CpG islands in Vertebrates). There is no upper cut to exclude large fragments as they tend to be lost in the processing of the different steps of the protocol.

Illumina has stated that the sequences of combined indexes matters and that there should be as much diversity of the 4 bases as possible for the combined adapters. Your protocol seems to be more concerned with combining samples with like Ct values, and less concerned with diversity of index sequence. Could it lead to any issues?

It is right that in our pooling protocol the priority is given to a balanced representation of all samples and good homogeneity in order to make sure that after bisulfite conversion, amplification and sequencing, every sample in the pool is getting enough and homogenous number of reads. We know good balance in the index is also important but it is mainly a point of concern when a small number of samples are multiplexed (less than 4). When multiplexing 4 samples or more, as it is often the case with RRBS, you can use any combination of indexes.

How many sequencing reads do you recommend per sample to get the necessary depth?

For human or mouse, we recommend aiming to get about 30-40M raw reads per sample. It is always better to have a high sequencing depth but it has a cost. We have estimated that this sequencing depth is a good balance between price and coverage. In Human, this permits to collect ~4 M CpGs with a mean coverage >10X after PCR duplicates removal from 100 ng gDNA samples pooled by 5.

Do we have to use special sequencing kits or primers?

No, the RRBS libraries are compatible with classical Illumina sequencing kits and primers. However, to allow UMI sequence reading during sequencing, it is necessary to specify a particular run mode/recipe to your sequencing provider for RRBS-UDI libraries. UMI sequence is following the i7 sequencing, thus the appropriate run mode is : cycle 50-8-(i5) and 17-(i7)-50. As a consequence, you should ask for delivery of the reads unattributed and undemultiplexed and perform the indexes and UMI processing yourself following our data analysis pipeline recommendations and tools (see section Data Analysis Recommendations).

Is your kit designed for directional sequencing?

Yes, our RRBS protocol is directional.

I am also interested in 5-hmC, is your RRBS kit compatible with oxBS or TAB-seq?

Theoretically it is possible to do oxBS or TAB-seq in combination with our Premium RRBS Kit, however, we have not yet validated those applications.

Related Products

Product	Cat. No.
Premium Methyl UDI-UMI Adapters Modules – Set A	C02030040
Premium Methyl UDI-UMI Adapters Modules – Set B	C02030041
Bisulfite conversion reagent for RRBS	C02030035
DNA methylation control package V2	C02040019
MethylTaq Plus 2X Master Mix	C09010012
MicroChIP DiaPure columns	C03040001

Revision history

Version	Date of modification	Description of modifications
Version 2 04_2024	April 2024	Correction of the Primer Mix volume.
Version 2 03_2024	March 2024	Addition of the new product (Premium Methyl UDI-UMI Adapters – 96 rxns) and related information
Version 2 01_2024	January 2024	1. Page 37: Addition of the bcl2fastq command and instructions to obtain the Undetermined fastq files. 2. Page 38: More detailed explanations for the demultiplexing using fumi_tools. 3. Page 41: Addition of bcl2fastq in the list of useful tools. 4. Addition of more pooling information
Version 2 08_2022	August 2022	Addition of the new kit format (96 rxns) and related information
Version 2 06_2022	June 2022	1. Page 26 - Update of the instructions for the use of the SIP 2. Page 36 - Update of sequencing recommendations 3. Addition of a note regarding spike-in amounts in low multiplexing experiments 4. Removal of the table 5
Version 2 03_2022	March 2022	1. Update of storage temperatures 2. Simplification of the instructions for the use of multichannel pipettes 3. Removal of transition step of adapters in 0,2 mL tubes at Step 2 4. Addition of the reminder - step 3.4 5. Re-phrasing of step 3.6 and 4 6. Suppression of Intermediary dilution step at the step 4 7. Suppression of holding steps in the qPCR programs 8. Update of the instructions for the use of the SIP 9. Addition of notes - Step 5, Step 7.1 10. Addition of a second elution after step 5.14 11. Correction of timing for step 6 12. Typos correction

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