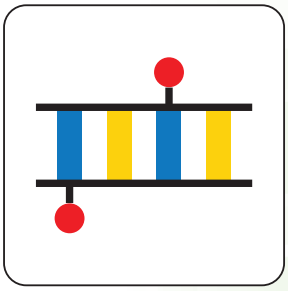
USER GUIDE



Ovation® RRBS Methyl-Seq System 1–16

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Revision: v7

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II. Introduction

A. Background

Reduced representation bisulfite sequencing (RRBS) is a technique used to generate single base resolution DNA methylation (5-methylC) information across a genomic sample. By analyzing a reduced representation of the genome, the amount of sequencing required is greatly reduced relative to whole genome bisulfite sequencing. Since the RRBS technique was first described (Meissner, et al. (2005) *Nucleic Acids Res* 33(18):5868), there have been many enhancements and improvements (see for example, Boyle, et al. (2012) *Genome Biol* 13:R92). The current approach utilizes the methylation insensitive restriction enzyme MspI, which recognizes CCGG. As a result of partial fragmentation during bisulfite conversion, PCR, and efficiency of cluster generation, only a subset of these fragments, typically under 300 bp in length, are sequenced.

Figure 1 is a Bioanalyzer trace of MspI digested human genomic DNA. Note how only a small amount of the digested DNA is represented in the under 300 bp fraction. However these smaller fragments are derived from genomic DNA that has a high frequency of MspI sites and therefore a high frequency of potential CpG methylation sites. This is how RRBS achieves reduced representation.

Figure 1. Bioanalyzer trace of MspI digested human genomic DNA.

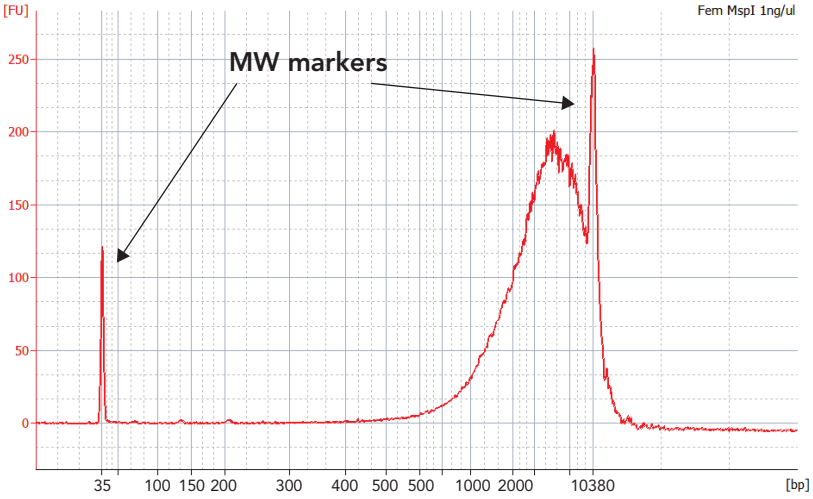
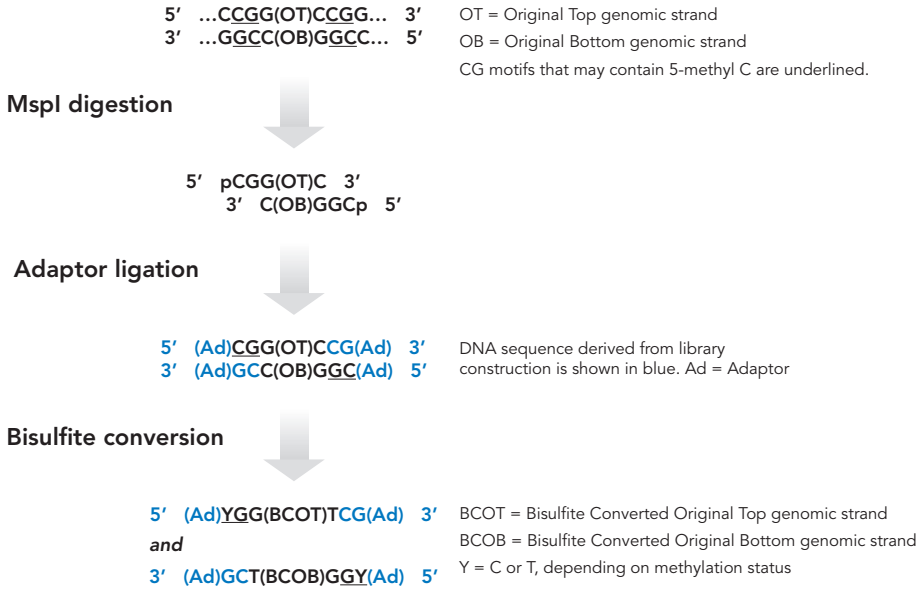


Figure 2 follows a hypothetical MspI fragment as it is processed with the Ovation RRBS Methyl-Seq System. The digested fragments are directly ligated to cohesive-ended adaptors, without the requirement for blunting or A-tailing. This both simplifies the workflow and increases efficiency. MspI enzyme is still active during ligation. Therefore, any genomic fragments that ligate to each other will be re-cleaved. Since adaptor ligation does not recreate an MspI site, these desired ligation products are stable.

I. Introduction

Figure 2. MspI fragment during processing with the Ovation RRBS Methyl-Seq System 1–16.

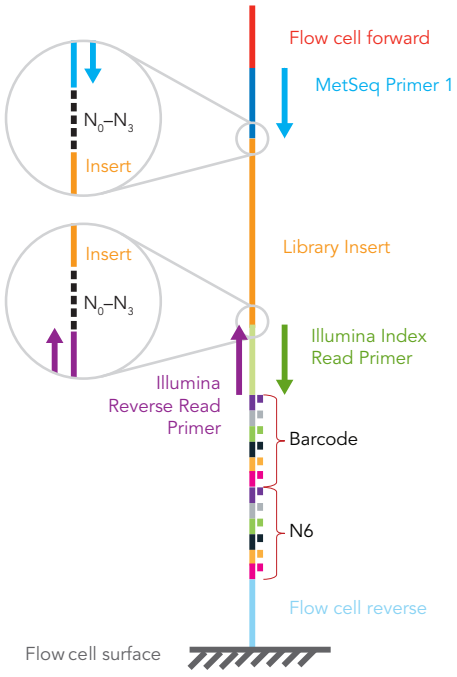


Two library molecules from each fragment are possible, one from each strand. Note that after bisulfite conversion, they are non-complementary. Also note that for each MspI fragment, the methylation information for the CpG in the MspI site to the left is preserved in the library molecule derived from the top strand, whereas the methylation information for the CpG in the MspI site to the right is preserved in the library molecule derived from the bottom strand.

Traditionally, PCR duplicates are identified in libraries made from randomly fragmented inserts by mapping inserts to the genome and discarding any paired end reads that share the same genomic coordinates. This approach doesn't work for restriction digested samples, such as RRBS, because all fragments mapping to a genomic location will share the same ends. Tecan provides a way to identify unique molecules in RRBS libraries by adding an additional 6 bases of random sequence after the 6 base barcode. See **Figure 3** for a schematic of the Ovation RRBS Methyl-Seq System library molecule. If you wish to utilize this PCR duplicate marking feature, increase the index read from 6 to 12 nucleotides, then use the Tecan-provided Duplicate Marking tool, NuDup, to identify and discard any PCR duplicates found. The tool works by looking at the random N6 sequence associated with all reads that map to the same genomic location. Any reads that both map to the same genomic location and contain the same N6 sequence are considered duplicates, and all but one copy are removed.

I. Introduction

Figure 3. Ovation RRBS Methyl-Seq System 1–16 library molecule schematic.



During sequencing on Illumina instruments, software identifies clusters over the first several cycles of sequencing. During sequencing of normal, high diversity libraries, overlapping clusters can be distinguished because they are different colors. If overlapping clusters contain the same sequence during the first few cycles, they may be mistaken as a single cluster. When the two sequences eventually diverge in a mixed cluster, low quality and/or incorrect base calls are the result. In the RRBS strategy, all reads begin with either CGG or TGG, depending on methylation state. This makes sequencing traditional RRBS libraries on Illumina instruments challenging. The Ovation RRBS Methyl-Seq System 1–16 overcomes this challenge by inserting a variable number of random bases at the beginning of both forward and reverse reads. The variable number of bases dephases the clusters across the image tile such that all four bases are present in every cycle. The random nature of the sequences reduces the chance of overlapping clusters containing the same initial sequence and being miscalled as a single cluster. As shown in **Figure 4**, the added diversity is not completely random. Instead, it is carefully chosen such that it can be identified and removed from the sequencing read using the Tecan diversity trimming script. This preserves the first base of the MspI fragment, which contains a CpG methylation measurement. The RRBS approach results in a very efficient use of sequencing capacity, with more than 1 CpG measurement per read. The enhancements provided by the Ovation RRBS Methyl-Seq System 1–16 deliver the efficiency of RRBS sequencing with improved workflow, higher quality base calls at higher cluster density, and the ability to identify unique molecules.

Figure 4. Types of sequence diversity added to the beginning of forward reads.

Blue = DNA sequence derived from library construction

0 diversity bases added	5′	YGG(BCO)	**BCO** = Bisulfite-Converted Original genomic strand
1 diversity base added	5′	DYGG(BCO)	**Y** = C or T, depending on methylation status
2 diversity bases added	5′	DDYGG(BCO)	**D** = A, G, or T
3 diversity bases added	5′	RDDYGG(BCO)	**R** = A or G

The Ovation RRBS Methyl-Seq System 1–16 provides a simple, fast and scalable solution for producing directional reduced representation bisulfite-converted libraries for next generation sequencing.

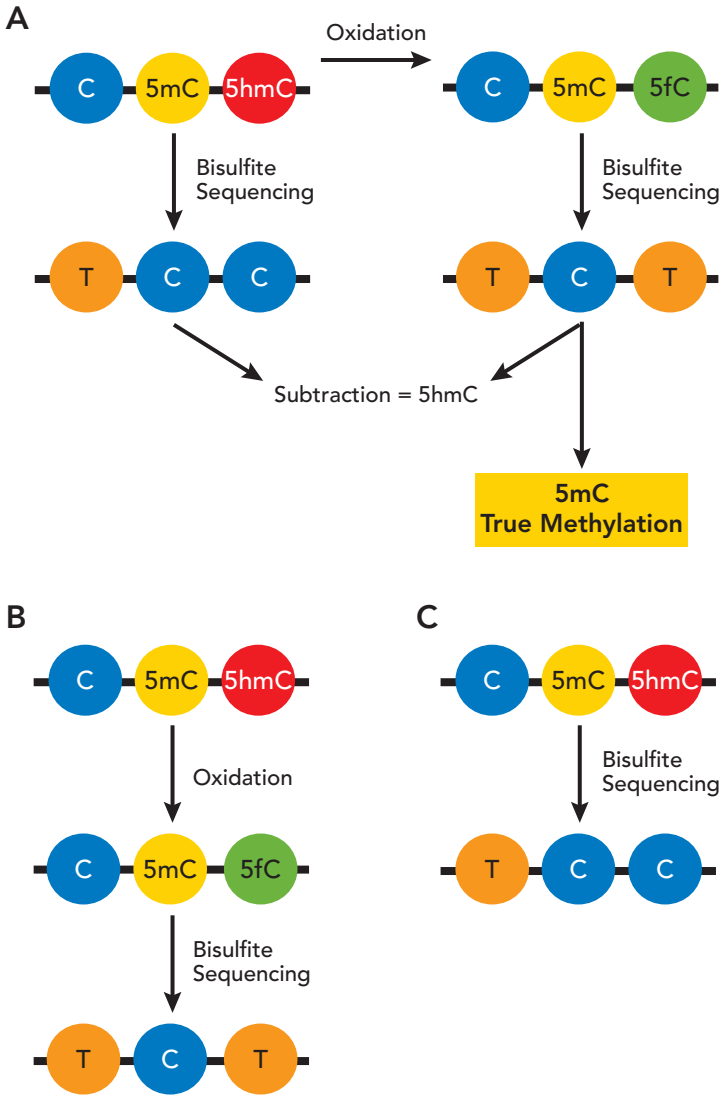
I. Introduction

TrueMethyl® oxBS module

The Ovation RRBS Methyl-Seq System features the TrueMethyl oxBS Module for the interrogation of both 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC), providing a method to accurately quantify the true level of cytosine methylation. The module contains the necessary reagents to perform the quantitative site-specific oxidation, and conversion to uracil, of 5hmC bases in a complex genomic sample (as outlined initially in Quantitative Sequencing of 5-Methylcytosine and 5-Hydroxymethylcytosine at Single-Base Resolution. Booth M.J. et al. *Science* 336, 934 (2012)). It also includes the necessary reagents for bisulfite conversion of the libraries.

Researchers interested in quantitating 5hmC can use the TrueMethyl oxBS Module to process 16 samples in parallel preparations of oxBS and bisulfite-only to determine the 5hmC content through subtractive analysis methods (**Figure 5A**). For those interested only in 5mC, there are sufficient reagents to process 32 individual samples through the oxBS workflow (**Figure 5B**). Alternatively, 32 samples may be processed through the bisulfite-only workflow for indiscriminate detection of 5mC and 5hmC (**Figure 5C**).

Figure 5. TrueMethyl oxBS Module workflow options.



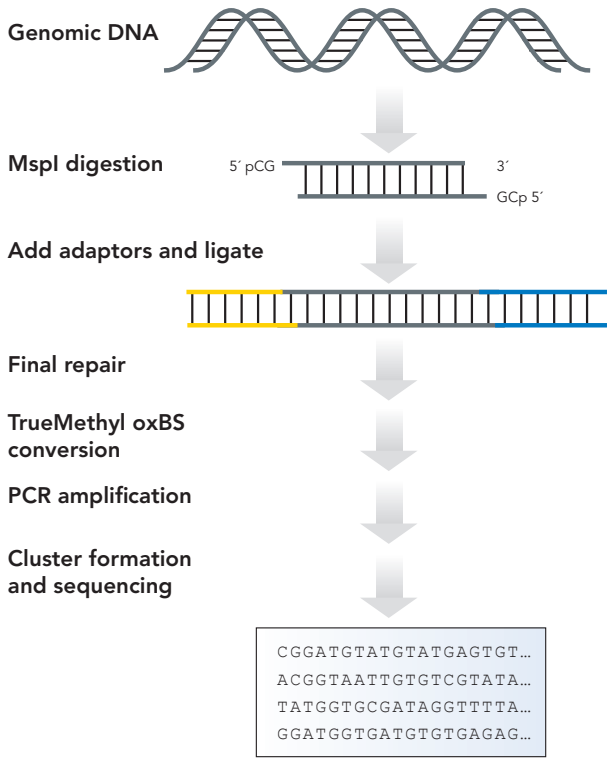
I. Introduction

The Ovation RRBS Methyl-Seq System is fully compatible with the TrueMethyl oxBS Module, and can be purchased as a bundle (Part No. 0553).

B. Workflow

As shown in **Figure 6**, the Ovation RRBS Methyl-Seq System features a streamlined workflow consisting of five main steps: MspI digestion, adaptor ligation, final repair, bisulfite conversion, and PCR amplification to produce the final library. With the TrueMethyl oxBS Module, an optional DNA oxidation can be performed prior to bisulfite conversion for analysis of 5hmC. The entire workflow can be completed in less than two days and yields DNA libraries ready for cluster formation and either single read or paired-end sequencing. The kit provides sufficient reagents to generate 32 libraries, two libraries each from 16 different barcodes. The barcodes have been carefully selected so that all 16 can be sequenced in eight color balanced duplexes. In addition, the barcodes are edit distance 3 from each other, which means they can be parsed with one mismatch, further increasing data yield per run.

Figure 6. Schematic of the Ovation RRBS Methyl-Seq System 1–16 workflow.



I. Introduction

C. Performance Specifications

The Ovation RRBS Methyl-Seq System 1–16 is designed to produce RRBS libraries from human genomic DNA suitable for either single read or paired-end sequencing on IIllumina NGS platforms without gel-based size selection, using 100 ng of high-quality human genomic DNA as input. This system generates libraries suitable for cluster generation in a single day.

D. Quality Control

Every lot of the Ovation RRBS Methyl-Seq System 1–16 undergoes functional testing to meet specifications for library generation performance.

E. Storage and Stability

Note: This product contains components with multiple storage temperature requirements. All shipments should be unpacked immediately upon receipt and stored as directed below.

Ovation RRBS Methyl-Seq System 1–16

The Ovation RRBS Methyl-Seq System 1–16 is shipped on dry ice and should be unpacked immediately upon receipt.

The vials labeled Agencourt® Beads (clear cap) should be removed from the top of the shipping carton upon delivery and stored at 4 °C.

The Ovation RRBS Methyl-Seq Core Kit (0353-32) should be stored at –20 °C on internal shelves of a freezer without a defrost cycle. The kit has been tested to perform to specifications after as many as six freeze/thaw cycles. Kits handled and stored according to the above guidelines will perform to specifications for at least six months.

TrueMethyl oxBS Module

The TrueMethyl oxBS Module is shipped in two separate boxes and separately from the Ovation RRBS Methyl-Seq System 1-16.

Box 1 is shipped at room temperature.

- The Magnetic Bead Solution, Binding Buffer 1 and Binding Buffer 2 should be removed from Box 1 and stored at 4 °C.
- All other buffers and the Bisulfite Reagent aliquots should be stored at room temperature.

Box 2 is shipped on frozen ice packs.

- It contains a single reagent, the Oxidant Solution, which should be stored at -20 °C.

***Important:** The Oxidant Solution is sensitive to carbon dioxide exposure. Under no circumstance should the oxidant come into contact with CO_2 or dry ice otherwise performance will be significantly impaired. The oxidant is shipped in Box 2 on frozen ice packs and not dry ice for this reason.

I. Introduction

F. Safety Data Sheet (SDS)

If appropriate, an SDS for this product is available on the NuGEN website at www.nugen.com/products/ovation-rrbs-methyl-seq-truemethyl-oxbs.

G. Before You Start

Please review this User Guide before using this kit for the first time, including the "Kit Components", "Planning the Experiment", "Overview", "Protocol" and "FAQ" sections. For more information, visit the Ovation RRBS Methyl-Seq page at NuGEN.com (www.nugen.com/products/ovation-rrbs-methyl-seq-truemethyl-oxbs). New to NGS? Contact Tecan NGS Technical Support at techserv-gn@tecan.com for tips and tricks on getting started.

II. Components

A. Reagents Provided

Table 1. Ovation RRBS Methyl-Seq System 1–16 reagents (Part No. 0353)*

DESCRIPTION	0353-32 PART NUMBER	VIAL LABEL	VIAL NUMBER
MspI Buffer Mix	S02137	Blue	—
MspI Enzyme Mix	S02138	Blue	—
Ligation Buffer Mix	S01534	Yellow	L1 ver 4
Ligation Adaptor Mix	S02140 S02141 S02142 S02143 S02144 S02145 S02146 S02147 S02148 S02149 S02150 S02151 S02152 S02153 S02154 S02155	Yellow	L2V20DR-BC1 L2V20DR-BC2 L2V20DR-BC3 L2V20DR-BC4 L2V20DR-BC5 L2V20DR-BC6 L2V20DR-BC7 L2V20DR-BC8 L2V20DR-BC9 L2V20DR-BC10 L2V20DR-BC11 L2V20DR-BC12 L2V20DR-BC13 L2V20DR-BC14 L2V20DR-BC15 L2V20DR-BC16
Ligation Enzyme Mix	S01535	Yellow	L3 ver 4
Final Repair Buffer Mix	S02139	Purple	FR1 ver 4
Final Repair Enzyme Mix	S01569	Purple	FR2
Amplification Primer Mix	S01739	Red	P2 ver 8
Amplification Enzyme Mix	S01740	Red	P3 ver 3
100 µM Sequencing Primer*	S02670	Orange	MetSeq Primer 1
Nuclease-free Water	S01001	Green	D1
Agencourt Beads	S01502	Clear	—
DNA Resuspension Buffer Mix	S02156	Clear	DR1

* **Note:** Please contact Tecan NGS Technical Support for reagent information on 96 reaction manual and automation kits.

II. Components

Table 2. TrueMethyl oxBS Module (Part No. 0414)

COMPONENT	VIAL NUMBER
Oxidant Solution	TM-03-013
Denaturing Solution	TM-03-003
Ultra Pure Water	TM-03-002
Bisulfite Reagent Aliquot	TM-03-004 (8)
Bisulfite Diluent	TM-03-007
Desulfonation Buffer Concentrate	TM-03-005
Elution Buffer	TM-03-011
Magnetic Bead Solution	TM-03-008
Binding Buffer 1	TM-03-009
Binding Buffer 2	TM-03-010

B. Additional Equipment, Reagents and Labware

Required Materials

- **Equipment**
  - Agilent 2100 Bioanalyzer or 2200 TapeStation Instrument, or other equipment for electrophoretic analysis of nucleic acids
  - Qubit® Fluorometer (Thermo Fisher Scientific) or appropriate fluorometer and accessories for quantification of fragmented DNA and amplified libraries
  - Microcentrifuge for individual 1.5 mL and 0.5 mL tubes
  - Microcentrifuge for 0.2 mL tube strips or plates
  - Variable speed microcentrifuge suitable for use with 1.5 mL microcentrifuge tubes and ability to set the rotor to speeds of 14000 x g (RCF)
  - 0.5–10 µL pipette, 2–20 µL pipette, 20–200 µL pipette, 200–1000 µL pipette
  - Vortexer
  - Thermal cycler with 0.2 mL tube heat block and heated lid
  - Heat block, thermomixer or heated orbital incubator able to maintain temperatures at 37 °C and 60 °C (e.g. Eppendorf® Thermomixer Comfort), for TrueMethyl oxBS Module
- **Reagents**
  - HPLC grade 100% Acetonitrile (Thermo Fisher Scientific, Cat. #A998-1), for TrueMethyl oxBS Module
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - Nuclease-free water (Alfa Aesar, Cat. #J71786), for purification steps
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793) optional; for diluting nucleic acids
  - OPTIONAL: EvaGreen® Dye, 20X in water (Biotium, Cat. #31000)

II. Components

- **Supplies and Labware**
  - Nuclease-free pipette tips with aerosol barriers
  - 0.2 mL polypropylene PCR tube strips* or 0.2 mL thin-wall polypropylene PCR plates*, nuclease-free
  - 0.5 mL and 1.5 mL polypropylene microcentrifuge tubes*, nuclease-free (DNA LoBind Tubes, Eppendorf, Cat# 0030108035 or 0030108051)
  - 15 mL and 50 mL polypropylene centrifuge tubes* (e.g. Corning™ Falcon™ tube))
  - Magnetic separation plate or rack for 0.2 mL strip tubes or plates (Beckman Coulter Cat. #A29164 or A32782; Thermo Fisher Scientific Cat. #12331D, 12027, or 12332D; Promega Cat. #V8351; others). Other magnetic stands may be used as well, although their performance has not been validated by NuGEN
  - Disposable gloves
  - Kimwipes
  - Ice bucket
  - Cleaning solutions such as DNA-OFF™ (MP Biomedicals, Cat. #QD0500)
  - OPTIONAL: PhiX Control (Illumina, Cat. #FC-110-3001)
  - OPTIONAL: Real-time PCR system

*__Important:__ Avoid use of "extra low adhesion" coated plastic consumables e.g. Protein Lo-bind tubes. These types of coated plastic consumables are NOT suitable for oxidation reactions.

To Order:

- Agilent, www.agilent.com
- Alfa Aesar, www.alfa.com
- Beckman Coulter, www.beckmancoulter.com
- Biotium, www.biotium.com
- Eppendorf, www.eppendorf.com
- MP Biomedicals, www.mpbio.com
- Qiagen, www.qiagen.com
- Promega, www.promega.com
- Sigma-Aldrich, Inc., www.sigmaaldrich.com
- Thermo Fisher Scientific, www.thermofisher.com

III. Planning the Experiment

A. Input DNA Requirements

DNA Quantity

The Ovation RRBS Methyl-Seq System 1–16 is designed to work with inputs of 100 ng of intact human genomic DNA. Quantitation of gDNA by a dsDNA assay, such as PicoGreen® or the Qubit System, is highly recommended.

DNA Purity

DNA samples must be free of contaminating proteins, RNA, organic solvents (including phenol and ethanol) and salts. We recommend using a commercially available system for gDNA isolation. The A260:A280 ratio for DNA samples should be in excess of 1.8. Using DNA samples with lower ratios may compromise your results.

DNA Integrity

Although not recommended, it is possible to generate RRBS libraries from less than 100 ng of gDNA, or from degraded gDNA, such as DNA extracted from formalin fixed, paraffin embedded (FFPE) specimens. Please contact Tecan NGS technical support (techserv-gn@tecan.com) for specific recommendations for using challenging samples and low inputs.

B. Preparation of Desulfonation Buffer for the TrueMethyl oxBS Module

1. Prepare a working solution of desulfonation buffer by combining 30% Desulfonation Buffer Concentrate and 70% ethanol (e.g. for 4 samples combine 300 µL of Desulfonation Buffer Concentrate and 700 µL 100% ethanol).
2. Seal lid tightly and mix thoroughly by vortexing or inversion.

Important:

- Prepare the Desulfonation Buffer at least 2 hours in advance of opening or using the Oxidant Solution. Exposure of the Oxidant Solution to alcohol vapor can cause irreversible decomposition of the Oxidant Solution, therefore care must be taken to avoid such exposure.
- Ensure the Desulfonation Buffer is prepared exactly as described, using fresh 100% ethanol. Failure to do so may result in gross loss of sample due to elution of the DNA from the beads during the Desulfonation step.

III. Planning the Experiment

C. Oxidation Solution Sensitivity

Important: The reactivity of this reagent allows the selective oxidation of 5-hmC to 5-fC. However, the oxidant will react with other contaminating species in solution if present. Reaction of the oxidant with anything but DNA will decrease the active concentration of oxidant in solution and lead to the possibility of suboptimal conversion and also generate products that could inhibit downstream steps of the workflow (e.g. bisulfite conversion and PCR amplification).

To minimize the likelihood of side reactions in the oxidation reaction, follow the instructions in this guide closely. It is critical that the input DNA samples have been prepared with the sensitivity of the oxidant in mind, adhering to the guidelines below as far as practically possible.

Solution Sensitivities

Contaminating compounds in solution known to be incompatible with the working Oxidant Solution:

- Alcohols (ethanol, isopropanol, phenol)
- Alcohol-containing compounds (Tris, EB buffer, TE buffer, glycerol, surfactants)
- Solutions < pH 9.0
- Carbon dioxide

Care should be taken to remove traces of such compounds from the DNA sample solutions prior to input into the TrueMethyl oxBS workflow. A buffer exchange step via magnetic bead purification is included in the protocol but the effectiveness of this exchange is dependent on the type and concentration of contaminating compounds in solution.

Environmental Sensitivities

The oxidation stock solution and working oxidation solution have been shown to react when exposed to certain compounds under specific environmental conditions. Care should be taken before removing the Oxidant Solution from its protective foil shipping bag; from the freezer in which it is stored; and before uncapping the tube to prevent exposure to these compounds.

- **Alcohol vapor.** Exposure of the Oxidant Solution to alcohol vapor (e.g. after wiping down a bench with 70% ethanol prior to experimentation) can cause irreversible decomposition of the Oxidant Solution. If it is your practice to clean surfaces in this manner, we recommend doing so at least 2 hours in advance of opening or using the Oxidant Solution as a precaution.
- **Carbon dioxide.** Exposure of the Oxidant Solution to high local concentrations of CO_2 in the air (e.g. leaving a polystyrene shipping cooler containing dry ice on a bench in the vicinity or standing the Oxidant Solution loosely capped on dry ice) can cause irreversible decomposition of the Oxidant Solution. It is strongly advised not to open or use the Oxidant Solution near a source of carbon dioxide. Some –80 °C freezers are purged with CO_2; for this reason we do not recommend storage of the Oxidant Solution or oxBox2 in a –80 °C freezer.

D. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

III. Planning the Experiment

E. Using the Ovation RRBS Methyl-Seq System on Illumina NGS Platforms

Libraries generated with the Ovation RRBS Methyl-Seq System 1–16 are compatible with Illumina NGS platforms. These libraries should be sequenced using the Illumina protocol for multiplex sequencing. The barcode sequences, found in Appendix A, must be entered into the Illumina software prior to analysis.

Important:

- The design of the Ovation RRBS Methyl-Seq System 1–16 requires the use of a custom Read 1 sequencing primer, MetSeq Primer 1, included in this kit at a concentration of 100 µM. Sequencing with custom primers may not be supported on all Illumina platforms. Please follow the custom primer recommendations for your specific sequencer. **Please note** that the concentration of the sequencing primer (MetSeq Primer 1) has changed. For additional information see page 8.
- The Standard Read 1 Primer is required when multiplexing with PhiX or other libraries. MetSeq Primer 1 should be mixed with the Standard Read 1 primer to sequence PhiX and/or other libraries. The standard Illumina primers are used for the index, and if desired, reverse reads.

The barcode sequences used in this kit were carefully chosen for their ability to parse properly and for color balancing. Therefore, strict requirements exist for choosing barcoded libraries for a multiplexed sequencing run. Refer to Appendix A., Table 11 for multiplexing guidelines.

If you wish to use the Duplicate Marking feature built into the Ovation RRBS Methyl-Seq System, add an additional 6 nt to the index read. For more information on this feature, see Appendix D. Data Analysis of Ovation RRBS Methyl-Seq Libraries.

The Ovation RRBS Methyl-Seq System 1–16 produces directional bisulfite-converted libraries. This means Read 1 will sequence the C-to-T converted strand and the overall nucleotide balance for Read 1 will show a low proportion of C bases. Illumina has recommendations for how to obtain high-quality base calls from libraries containing unbalanced nucleotide ratios. For the highest quality results, follow the low-diversity sequencing recommendations for your specific instrument. If you are sequencing on a HiSeq or MiSeq, make sure your instrument is running the following software versions or later:

- HiSeq — HCS v2.2.38 (includes RTA v1.18.61)
- MiSeq — MSR 2.6.2 or higher (RTA v1.17.28)

These sequencing recommendations, in combination with the sequence diversity incorporated in the Ovation RRBS Methyl-Seq System adaptors, can produce high-quality RRBS reads on compatible platforms without the need to spike in balanced library such as PhiX. However, spiking in 5% PhiX or another previously characterized, high-quality balanced library can be useful for troubleshooting purposes in the event of a failed run, while only reducing the data output by 5%. Such a control can be used to tell if the sequencing run failed in general, or if there is a problem specific to the particular RRBS library being sequenced. Please refer to the Illumina technical support for your instrument for more information.

One of the principal benefits of the RRBS method is that the first base of the read contains a CpG methylation measurement. In principle, reads need only be long enough to accurately map them to the genome. However, mapping rates are directly affected by sequence read length, and use of longer reads can lead to more uniquely mapping reads and coverage of a greater number of CpG loci.

F. Index Structure and Index Read Recommendations

The RRBS System uses 6-base barcodes for sample multiplexing. The 6-base barcode sequences can be found in Appendix A. In addition to the 6-base barcode, the adaptor contains 6 random bases immedi-

ately following the 6-base barcode, for a total of 12 bases. The additional 6 bases are used for duplicate read determination using the Duplicate Marking Tool (see section D. Data Analysis of Ovation RRBS Methyl-Seq Libraries). To take advantage of this feature, the libraries should be sequenced using 12 cycles for the index read.

Parsing and Generating an index.fastq File For De-duplication

Illumina does not provide a simple way to obtain the sequence information contained in the 12-base pair index read including the 6 random bases that are necessary for duplicate read determination. Several recommended methods to generate the necessary index fastq file are provided below.

MiSeq Instrument

Parsing multiplex runs using the MiSeq built-in Illumina software replaces the barcode sequence from each library with a numerical substitute, which removes the duplicate information provided by the N6 sequence present after the barcode. To retrieve this information using the MiSeq instrument use one of the options given below.

Option 1: (Recommended) contact Illumina Technical Support and request a modification of the MiSeq config file to allow generation of an index fastq file during data analysis.

Option 2: Modify the MiSeq config file to allow generation of an index fastq file during data analysis:

Stop the MiSeq Reporter process.

Locate the "MiSeq Reporter.exe.config" file located in C:/Illumina/MiSeq Reporter

Open config file and search for a line that reads:

<add key="CreateFastqForIndexReads" value="0"/>

If this line is present, change the value from "0" to "1".

If this line is not present, add the line to the config file using the add keys function under the appSettings tab with the value set as "1".

Restart the MiSeq reporter process.

Re-queue the run for data analysis if required. The 6-base barcodes followed by NNNNNN should be entered into the sample sheet to enable proper multiplex library parsing.

HiSeq and NextSeq Instruments

Note: Older versions of the sequencer software may have incompatibilities with a 12 base index read. It is recommended that the HiSeq software be updated to the most recent version.

When setting up a sequencing run, specify 12 bases of index sequencing (no sample sheet is required). However, if you wish to include a sample sheet, specify only the 6 bases of the actual barcode. Do not include the N6 in your sample sheet. Use the method described below to parse and generate the N6 index fastq files for HiSeq and NextSeq instruments using bcl2fastq2 version 2.17.

Browse to the location of the run folder (called "RunFolder" in this example).

Run bcl2fastq2. Use the "`--use-bases-mask Y*,I6Y*`" option to generate an Index fastq file along with the forward read (for paired end reads use "--use-bases- mask Y*,I6Y*, Y*"). For example:

III. Planning the Experiment

```
/usr/local/bin/bcl2fastq --runfolder-dir . --output-dir ./ Data/Intensities/
BaseCalls/--no-lane-splitting --sample-sheet SampleSheet.csv --use-bases-mask
Y*,I6Y* --minimum-trimmed-read- length 0 --mask-short-adapter-reads 0
```

Note: To generate the read and index fastq files without parsing, modify the `–use-bases-mask` option to "`--use-bases-mask Y*,Y*`". The generated fastq files can then be parsed using alternative software. In this case, the index fastq file will be labeled "`R2`" rather than "`I1`".

For information on sequencing with other sequencers, including NovaSeq, please contact Tecan NGS Technical Support.

G. Data Analysis and Parsing Multiplex Libraries

For the Ovation RRBS Methyl-Seq System 1–16, follow the recommendations in the Illumina technical support documentation on parsing barcodes. The sequences of the Ovation RRBS Methyl-Seq System 1–16 barcodes must be entered prior to parsing (the sequences are listed in Appendix A). With bisulfite-converted libraries, we notice a slightly higher rate of unmatched barcodes relative to non-bisulfite-converted libraries.

However, because the Ovation RRBS Methyl-Seq System uses edit distance 3 barcodes, you can allow one mismatch during parsing. This will significantly reduce the fraction of unmatched barcode reads.

Once the data have been parsed according to sample index, the reads must be trimmed before attempting alignment. Trimming is done in two steps. First, any low-quality bases and adaptor sequences are removed from the 3´ end, then the sequence diversity provided by the Ovation RRBS Methyl-Seq System adaptor is removed. At this point reads are ready for downstream analysis, such as mapping to the genome and determining methylation status. Please see Appendix D for recommendations on trimming, alignment and optional duplicate removal for unique molecule identification.

IV. Overview

A. Overview

The library preparation process used in the Ovation RRBS Methyl-Seq System 1–16 is performed in the following stages:

		TrueMethyl oxBS Workflow
1.	DNA digestion with MspI	1.0 hour
2.	Adaptor ligation	0.75 hours
3.	Final repair	0.25 hours
4.	DNA oxidation	2.0 hours
5.	Bisulfite conversion	2.0 hours
6.	Desulfonation and purification	1.5 hours
7.	Amplification and purification	1.5 hours
	Total time to prepare amplified library	**9.0 hours**

Components in the Ovation RRBS Methyl-Seq System 1–16 are color coded, with each color linked to a specific stage of the process. Performing each stage requires making a master mix, then adding it to the reaction, followed by incubation. Master mixes are prepared by mixing components provided for that stage.

It is important to produce each library independently, and not to mix adaptors during the actual library construction protocol. Samples should be multiplexed by mixing the amplified libraries prior to cluster formation. The barcode sequences in this kit were carefully chosen for their ability to parse properly and for color balancing. Therefore, strict requirements exist for multiplexing. Refer to Appendix A, Table 11 for multiplexing guidelines.

B. Protocol Notes

Controls

- We recommend the routine use of a positive control DNA, especially the first time a reaction is set up. The use of a positive control DNA will establish a baseline of performance and provide the opportunity to become familiar with the bead purification step. This step may be unfamiliar to many users and can be especially prone to handling variability in using the magnet plate, so a practice run with the plate is highly recommended.
- Routine use of a no template control (NTC) is recommended to monitor the work environment for potential carryover contamination of previous libraries.

General Workflow

- Set up no fewer than 4 reactions at a time to ensure that you are not pipetting very small volumes and to ensure sufficient reagent recoveries for the full nominal number of amplifications from the kit. Making master mixes for fewer than 4 samples at a time may affect reagent recovery volumes.
- Thaw components used in each step and immediately place them on ice. Always keep thawed reagents and reaction tubes on ice unless otherwise instructed. Do not thaw all reagents at once.

IV. Overview

- After thawing and mixing buffer mixes, if any precipitate is observed, re-dissolve the precipitate completely prior to use. You may gently warm the buffer mix for 2 minutes at room temperature followed by brief vortexing.
- Keep enzyme mixes on ice after briefly spinning to collect the contents. Do not vortex enzyme or adaptor mixes nor warm any enzyme, adaptor or primer mixes.
- When preparing master mixes, use the minimal amount of extra material to ensure you are able to run the maximum number of reactions using the components provided in the kit.
- When placing small amounts of reagents into the reaction mix, pipet up and down several times to ensure complete transfer from the pipet tip into the reaction mix.
- When instructed to mix via pipetting, gently aspirate and dispense a volume that is at least half of the total volume of the reaction mix.
- Always allow the thermal cycler to reach the initial incubation temperature prior to placing the tubes or plates in the block.

Reagents

- Use the water provided with the kit (green: D1) or an alternate source of nuclease-free water. We do not recommend the use of DEPC-treated water with this protocol.
- Components and reagents from other Tecan products should not be used with this product.
- Use only fresh ethanol stocks to make ethanol for washes in the purification protocols. Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the cDNA and wash it off the beads or column.

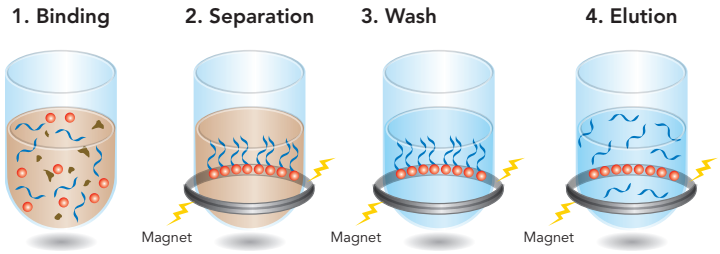
C. Magnetic Beads

Magnetic beads (Agencourt Beads and Magnetic Bead Solution) are provided for use with the Ovation RRBS Methyl-Seq Library System and TrueMethyl oxBS Module. You must follow the protocols outlined in this user guide for the use of these beads. The bead purification process used for DNA purification before amplification consists of:

- Binding of DNA to beads
- Magnetic separation of beads from supernatant
- Wash of bound beads to remove contaminants
- Elution

IV. Overview

Figure 7. Magnetic bead purification



Reproduced from original picture from Agencourt/Beckman Coulter Genomics

Tips and Notes

- Remove beads from 4 °C and leave at room temperature for at least 30 minutes prior to use. Cold beads and buffer will result in reduced recovery.
- Prior to use, ensure beads are fully resuspended by vortexing or inverting and tapping the tube.
- Note that our recommendations in the bead protocols may differ from the standard magnetic bead protocols. Please follow the protocol as written in this guide.
- If using strip tubes or partial plates, ensure they are firmly placed on the magnetic plate. The use of individual tubes is not advised as they are not very stable on the magnetic plates.
- It is critical to let the beads separate on the magnet for the full time indicated at each step. Removing the binding buffer before the beads have completely separated will impact yields.
- After the binding step has been completed, take care to minimize bead loss when removing the binding buffer. Loss of beads at this step may impact yields. With the samples placed on the magnetic plate, carefully remove the specified quantity of binding buffer from each sample to avoid disturbing the beads.
- Ensure that the 70% ethanol wash and 80% acetonitrile wash are freshly prepared from fresh stocks. Lower percent mixes will reduce recovery.
- During the washes, do not allow the beads to disperse. Keep the samples on the magnet in order to keep the beads on the walls of the plate wells or tubes.
- It is critical that all residual ethanol or acetonitrile be removed prior to elution. Therefore, when removing the final wash, first remove most of the supernatant, then allow the excess to collect at the bottom of the tube before removing the remaining supernatant. This also reduces the required bead air-drying time.
- After drying the beads, inspect each tube carefully and make certain that all the ethanol or acetonitrile has evaporated before proceeding with the next step.

IV. Overview

D. Programming the Thermal Cycler

Use a thermal cycler with a heat block designed for 0.2 mL tubes and equipped with a heated lid. Prepare the programs shown in Table 3 following the operating instructions provided by the manufacturer. For thermal cyclers with an adjustable heated lid, set the lid temperature at 100 °C only when sample temperature reaches above 30 °C. For thermal cyclers with a fixed-temperature heated lid, use the default settings (typically 100 to 105 °C).

Table 3. Thermal Cycler Programming

MspI DIGESTION		VOLUME
Program 1 MspI Digestion	37 °C – 60 min, hold at 4 °C	10 µL
LIGATION		
Program 2 Adaptor Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	20 µL
FINAL REPAIR		
Program 3 Final Repair	60 °C – 10 min, 70 °C – 10 min, hold at 4 °C	40 µL
OXIDATION		
Program 4 Denaturation	37 °C – 5 min, hold at 25 °C	10 µL
BISULFITE CONVERSION		
Program 5 Bisulfite Conversion	95 °C – 5 min, 60 °C – 20 min, 95 °C – 5 min, 60 °C – 40 min, 95 °C – 5 min, 60 °C – 45 min, hold at 20 °C	40 µL
AMPLIFICATION		
Program 6 Library Amplification	95 °C – 2 min, N(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s), hold at 10 °C	50 µL

Note: qPCR is recommended to determine the appropriate number of PCR cycles. For more information, contact Tecan NGS Technical Support.

V. Protocol

For each section of the protocol, remove reagents from storage as listed. Thaw and place reagents at room temperature or on ice as instructed. After each section, continue immediately to the next section of the protocol unless otherwise directed.

For protocol information on 96 reaction manual and automation kits, please contact Tecan NGS Technical Support at techserv-gn@tecan.com.

Important: Ensure a working stock of desulfonation buffer is prepared fresh at least 2 hours before use of the oxidant.

A. Sample Preparation

This protocol includes an integrated oxidative bisulfite workflow using the TrueMethyl oxBS Module.

- For each genomic DNA sample to be analyzed for 5hmC, two independent NGS libraries should be prepared. For each sample, one aliquot (100 ng) will be processed through oxBS and the other aliquot (100 ng) will be processed through a parallel MOCK oxBS workflow. The MOCK oxBS workflow excludes the oxidant solution.
- In order to multiplex the oxBS and MOCK oxBS processed samples, unique indexes are required.
- For bisulfite conversion without oxidation, follow the MOCK oxBS workflow.
- For alternate workflows without the TrueMethyl oxBS Module, substitute the 3rd party bisulfite treatment for steps V. E. – V. H. in the protocol. Step V.J. Library Amplification is designed to accommodate a volume of 20 µL of bisulfite converted, desulfonated DNA for PCR enrichment.

1. Aliquot each input sample (100 ng) into a 0.2 mL PCR strip tube or plate.
2. Adjust each sample to 8.5 µL with nuclease-free water.

B. MspI Digestion

Table 4. MspI Master Mix

REAGENT	MspI BUFFER MIX (BLUE)	MspI ENZYME MIX (BLUE)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	1.0 µL	0.5 µL

1. Spin down the MspI Enzyme Mix and place on ice.
2. Thaw MspI Buffer Mix at room temperature. Mix MspI Buffer Mix by vortexing, spin and place on ice.
3. Prepare a master mix by combining MspI Buffer Mix and MspI Enzyme Mix in a 0.5 mL capped tube, according to the volumes shown in Table 4. Mix by pipetting, spin down briefly and immediately place on ice.
4. Add 1.5 µL of MspI Master Mix to each sample tube for a total of 10 µL. Mix by pipetting, spin down and place on ice.

V. Protocol

5. Place the tubes in a thermal cycler programmed to run Program 1 (MspI Digestion; see Table 3):

   37 °C – 60 min, hold at 4 °C

6. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

C. Adaptor Ligation

Table 5. Ligation Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1)	LIGATION ENZYME MIX (YELLOW: L3)
STORAGE	—	–20 °C	–20 °C
1X REACTION VOLUME	2 µL	4 µL	1.0 µL

1. Spin down L3 and place on ice.
2. Thaw Adaptor Mixes (L2V20DR) on ice, spin down, and return to ice.

Important: Do not warm Ligation Adaptor Mixes above room temperature. Heating will severely degrade performance.

3. Thaw L1 at room temperature. Mix by vortexing, spin and place on ice.

Note: L1 is extremely viscous. Pipet this reagent slowly and mix thoroughly. Ensure it is well mixed after thawing, and that the Ligation Master Mix and ligation reactions are well-mixed.

4. Add 3 µL of the appropriate L2 Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting.
5. Just prior to use, prepare a master mix by combining D1, L1 and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 5. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use the master mix immediately.
6. Add 7 µL Ligation Master Mix to each reaction tube for a total of 20 µL. Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 2 (Ligation; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C

8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

V. Protocol

D. Final Repair

1. Remove the TrueMethyl oxBS Module Magnetic Bead Solution, Binding Buffer 1 and Binding Buffer 2 from 4 °C and place at room temperature for use in the next step.

Table 6. Final Repair Master Mix

REAGENT	FINAL REPAIR BUFFER MIX (PURPLE: FR1 VER 4)	FINAL REPAIR ENZYME MIX (PURPLE: FR2)	NUCLEASE-FREE WATER (GREEN: D1)
STORAGE	–20 °C	–20 °C	—
1X REACTION VOLUME	6 µL	0.5 µL	13.5 µL

2. Spin down FR2 and place on ice.
3. Thaw FR1 at room temperature. Mix by vortexing, spin down and place on ice.
4. Prepare a master mix by combining FR1, FR2 and D1 in a 0.5 mL capped tube according to the volumes shown in Table 6.
5. Add 20 µL of the Final Repair Master Mix to each sample for a total of 40 µL. Mix by pipetting, spin down and place on ice.
6. Place the tubes in a thermal cycler pre-heated to 60 °C and programmed to run Program 3 (Final Repair; see Table 3):

   60 °C – 10 min, 70 °C – 10 min, hold at 4 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.
8. If using the True Methyl Module, continue with section E. DNA Purification and Denaturation. For alternative bisulfite conversion methods, perform bisulfite conversion according to the manufacturer recommendations and proceed to protocol section I. Library Amplification Optimization with qPCR.

Important: Bisulfite conversion is critical for successful library amplification.

V. Protocol

E. DNA Purification and Denaturation

1. Remove the TrueMethyl oxBS Module Oxidant Solution from –20 °C and thaw on ice for use in the next step.

Note: The Oxidant Solution is light-sensitive. Keep protected from light.

Table 7. Magnetic Bead Binding Solution 1 Master Mix

REAGENT	BINDING BUFFER 1	MAGNETIC BEAD SOLUTION
STORAGE	4 °C	4 °C
1X REACTION VOLUME	120 µL	2.4 µL

2. Remove Acetonitrile, Ultra Pure water, and Denaturing Solution from storage and place on bench top.
3. Prepare a fresh stock of 80% acetonitrile, using the Ultra Pure water provided with the kit. Mix by vortexing or inversion and place at room temperature.
4. Ensure Magnetic Bead Solution and Binding Buffer 1 have reached room temperature before proceeding.
5. Mix Binding Buffer 1 by inversion until homogenized.
6. Vortex Magnetic Bead Solution until homogenized.
7. Prepare a master mix of Magnetic Bead Binding Solution 1 (MBBS1) as directed in Table 7.

Note: MBBS1 should be prepared fresh on the day of use. Do not store for longer than 1 week.

8. Vortex MBBS1 master mix thoroughly to ensure the beads are homogenized in solution.
9. At room temperature, add 10 µL of Ultra Pure water to each sample for a total of 50 µL.
10. Add 100 µL of MBBS1 master mix to each 0.2 mL tube containing 50 µL sample for a total of 150 µL. Mix by pipetting and centrifuge briefly.
11. Incubate at room temperature for 20 min.
12. Transfer tubes to a magnetic separation plate and incubate at room temperature for 5 minutes to completely clear the solution of beads.
13. Keeping the tubes on the magnet, carefully remove the supernatant and discard it.
14. With the tubes still on the magnet, carefully add 200 µL of 80% Acetonitrile wash to the tubes without disturbing the bead pellet.
15. Remove and discard the 200 µL 80% Acetonitrile wash, carefully avoiding aspiration of the bead pellet.

V. Protocol

16. Repeat Steps 13 and 14 twice to perform 3 x 200 µL 80% Acetonitrile washes in total. Remove as much of the final wash as possible.
17. Air dry the bead pellets for 5 minutes at room temperature, leaving the lids of the tubes open.

> **Note:** Ensure the tubes are dry before continuing the protocol. If the tubes aren't dry after 5 minutes, incubate for a longer period of time.

18. Remove the tubes from the magnet.
19. Add 10 µL of Denaturing Solution directly onto the bead pellet. Mix thoroughly to ensure all beads are resuspended.
20. Centrifuge briefly to collect solution at bottom of the tubes.
21. Place the tubes in a pre-warmed thermal cycler programmed to run Program 4 (Denaturation; see Table 3):

    37°C – 5 min, hold at 25 °C
22. Remove the tubes from the thermal cycler, spin to collect condensation and transfer to the magnet.
23. Incubate at room temperature for 2 minutes.
24. Carefully remove 9 µL of the eluate, ensuring as few beads as possible are carried over, transfer to a fresh 1.5 mL microcentrifuge tube and place at room temperature.

F. DNA Oxidation

1. Remove Ultra Pure water from storage and place on bench top.
2. Set a heat block to 40 °C.
3. Prepare individual oxidation and mock oxidation reactions as follows:
   - For each sample to be processed through the oxBS workflow, add 1 µL of oxidant solution to 9 µL of DNA for a total of 10 µL.
   - For each sample to be processed through the MOCK oxBS workflow, add 1 µL of Ultra Pure water to 9 µL of DNA for a total of 10 µL.
4. Mix reactions by vortexing and centrifuge briefly.
5. Place tubes in heat block and incubate for 10 min at 40 °C.
6. Centrifuge reactions at 14000 x g for 10 minutes at room temperature to pellet any black precipitate. MOCK oxBS treated samples will remain clear and will not have any black precipitate.

> **Important:** In samples treated with oxidant solution, the color of the oxidation reaction should remain orange after the 10 minute centrifugation, indicating a successful oxidation. If the solution turns any color other than orange, please see Appendix C.

V. Protocol

7. Transfer the orange supernatant to a fresh 0.2 mL PCR tube and place at room temperature. Proceed immediately to the next step.

Note:
- Take care not to carry any black precipitate over as this could inhibit downstream steps.
- Do not place the oxidized samples on ice to cool as this may cause the solution to precipitate.

G. Bisulfite Conversion

1. Set a heat block or heated orbital incubator to 60 °C.
2. Remove Bisulfite Diluent and Bisulfite Reagent aliquots from storage and place on bench top. Remove 1 aliquot of Bisulfite Reagent for every 20 reactions to be processed.
3. Prepare Bisulfite Reagent Solution by adding 700 µL of Bisulfite Diluent to each aliquot of Bisulfite Reagent.

Note: Each aliquot of Bisulfite Reagent Solution is sufficient for up to 20 samples. A fresh aliquot of solution should be prepared each time the kit is used and disposed of immediately after use.

4. Seal the lid of each aliquot with Bisulfite Reagent Solution tightly.
5. Incubate the aliquots of Bisulfite Reagent Solution for 15 min at 60 °C. Vortex regularly until the Bisulfite Reagent Solution is completely dissolved.
6. Spin down Bisulfite Reagent Solution briefly and place at room temperature.
7. Ensure oxidized DNA samples from previous step are at room temperature before proceeding.
8. Prepare Bisulfite Conversion Reaction mix by adding 30 µL of Bisulfite Reagent Solution to each 10 µL of DNA for a total of 40 µL. Ensure that each sample pair being processed through the oxBS and BS workflow is treated with the same aliquot of Bisulfite Reagent Solution.
9. Mix by pipetting, spin down and place at room temperature.

Note:
- If the Bisulfite Reagent Solution precipitates, return to 60 °C until dissolved.
- Samples treated with the oxidant solution may turn light gray in color.

10. Place the tubes in a pre-warmed thermal cycler programmed to run Program 5 (Bisulfite Conversion, see Table 3):

    95 °C – 5 min, 60 °C – 20 min, 95 °C – 5 min, 60 °C – 40 min, 95 °C – 5 min, 60 °C – 45 min, hold at 20 °C

Optional stopping point: You may hold samples at room temperature (+20 °C) for up to 16 hours. Do not store below +20 °C.

V. Protocol

11. Once the bisulfite conversion is complete, centrifuge samples briefly to collect solution at bottom of the tubes.
12. Transfer samples to 1.5 mL tubes and centrifuge for 10 min at 14000 x g.
13. Continue to H. Bisulfite-Converted DNA Desulfonation and Purification while the samples are in the centrifuge.

H. Bisulfite-Converted DNA Desulfonation and Purification

1. Remove Desulfonation Buffer, Binding Buffer 2, Magnetic Bead Solution and Elution Buffer from storage and place at room temperature for a minimum of 30 minutes before use.

Table 8. Magnetic Bead Binding Solution 2 Master Mix

REAGENT	BINDING BUFFER 2	MAGNETIC BEAD SOLUTION
STORAGE	4 °C	4 °C
1X REACTION VOLUME	200 µL	2.4 µL

2. Prepare a fresh stock of 70% Ethanol. Mix by vortexing or inversion.
3. Mix Binding Buffer 2 by inversion until homogenized.
4. Vortex Magnetic Bead Solution until homogenized.
5. Prepare a master mix of Magnetic Bead Binding Solution 2 (MBBS2) as directed in Table 8.

Note:
- MBBS2 should be prepared fresh on the day of use. Do not store for longer than 1 week.
- MBBS2 is a viscous solution. Pipet this reagent slowly and mix thoroughly. Ensure that MBBS2 and the MBBS2-sample mix are well-mixed.

6. Transfer 40 µL of the supernatant to a fresh set of 0.2 mL PCR tubes. Avoid disturbing the pellet in the oxidant solution-treated samples.
7. Vortex MBBS2 thoroughly to ensure the solution is homogenous before aliquoting.
8. Carefully add 160 µL of MBBS2 to each tube containing 40 µL bisulfite converted sample for a total of 200 µL. Mix thoroughly by pipetting slowly and gently, spin down and place at room temperature.
9. Incubate at room temperature for 5 minutes.
10. Centrifuge briefly to collect solution at bottom of the tubes.
11. Place the tubes onto the magnet and incubate at room temperature for at least 5 minutes to completely clear the solution of beads.
12. Carefully remove the supernatant and discard it.
13. Remove the tubes from the magnet.
14. Add 200 µL of 70% Ethanol to each sample tube. Resuspend the beads completely by pipetting.

V. Protocol

15. Place the tubes on the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.
16. Carefully remove the 70% Ethanol wash and discard it. Remove as much of the wash as possible.
17. Remove samples from the magnet.
18. Add 200 µL of Desulfonation Buffer with EtOH added directly onto the bead pellet. Resuspend the beads completely by pipetting.

Note: Be sure that the ethanol has been added to the desulfonation buffer, as described in Section III. B. Preparation of Desulfonation Buffer for the TrueMethyl oxBS Module.

19. Close lids of sample tubes securely and place the tubes into the magnetic separation rack. Incubate at room temperature for 5 minutes to completely clear the solution of beads.
20. Remove the tubes from the magnet, open the tubes, and return to the magnet.
21. Carefully remove 200 µL of the Desulfonation Buffer and discard it. Remove as much of the Desulfonation Buffer as possible without disturbing the bead pellet.
22. Remove the tubes from the magnet.
23. Add 200 µL of 70% Ethanol to each sample tube. Resuspend the beads completely by pipetting.
24. Place the tubes onto the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.
25. Remove the 200 µL 70% Ethanol wash and discard it.
26. Repeat Steps 23–25 to perform 2 x 200 µL 70% Ethanol washes in total. Remove as much of the final wash as possible.
27. Air-dry the beads on the magnet for 15 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated.
28. Remove the tubes from the magnet.
29. Add Elution Buffer directly onto the bead pellet and resuspend completely by pipetting:
    - For Library Amplification Optimization with qPCR (recommended), resuspend beads in 25 µL Elution Buffer.
    - If qPCR optimization is not required, resuspend beads in 21 µL Elution Buffer.
30. Incubate at room temperature for 5 minutes to elute the TrueMethyl converted DNA from the beads.
31. Centrifuge briefly to collect sample at bottom of the tubes.
32. Place the tubes onto the magnet and incubate at room temperature for 5 minutes to completely clear the solution of beads.
33. Carefully transfer eluate into a fresh 0.2 mL tube:
    - For Library Amplification Optimization with qPCR (recommended), transfer 24 µL eluate.
    - If qPCR optimization is not required, transfer 20 µL eluate.

V. Protocol

I. Library Amplification Optimization with qPCR

Note: qPCR optimization should be performed when running the kit for the first time, when using a new sample type or input, and any time degraded or low input samples are used.

Table 9. Library Amplification qPCR Master Mix

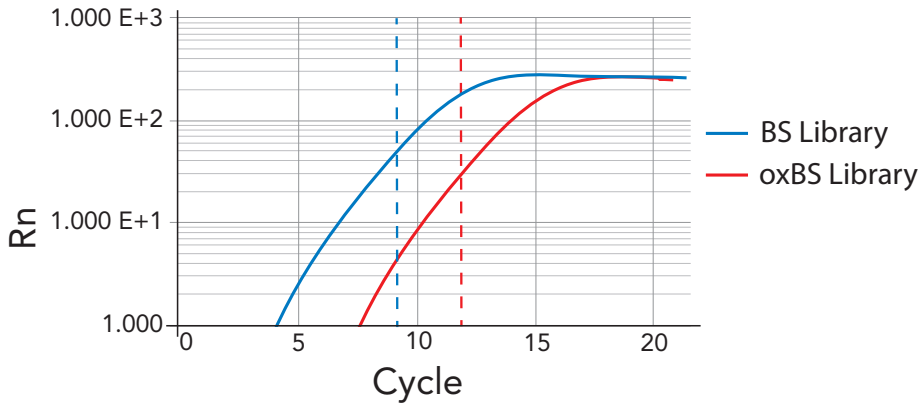
REAGENT	AMPLIFICATION PRIMER MIX (RED: P2 VER 8)	AMPLIFICATION ENZYME MIX (RED: P3 VER 3)	20X EvaGreen
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	1.0 µL	4.5 µL	0.5 µL

1. Prepare a master mix by combining P2, P3 and 20x EvaGreen in an appropriately sized capped tube according to the volumes shown in Table 9. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.
2. Aliquot 6 µL of master mix per sample into an appropriate PCR plate or optically clear strip tubes. Spin down and place on ice.
3. On ice, add 4 µL of sample to each 6 µL of Library Amplification qPCR Master Mix for a total of 10 µL per reaction. Reserve the remaining 20 µL of sample on ice.
4. Mix well by pipetting, spin down and place on ice.
5. Perform qPCR with the following cycling conditions:

   95 °C – 2 min, 30x(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s)
6. Examine the log fluorescence vs. cycle number plot from the qPCR system to determine the appropriate number of library amplification cycles.

   Select a cycle number within the middle to late exponential phase of the amplification plot. In the example in **Figure 8**, this is 9 cycles the 'BS Library' (blue dotted vertical line, left) and 12 cycles in the 'oxBS Library' (red dotted vertical line, right). Note the cycle number determined experimentally may differ from this stylized plot in **Figure 8**.

Figure 8. Stylized qPCR amplification plot



V. Protocol

J. Library Amplification

1. Remove Agencourt Beads from 4 °C and DR1 from –20 °C and place at room temperature for use in the next step.

Table 10. Library Amplification Master Mix

REAGENT	AMPLIFICATION PRIMER MIX (RED: P2 VER 8)	AMPLIFICATION ENZYME MIX (RED: P3 VER 3)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	5.0 µL	25.0 µL

Note: When starting with degraded DNA or significantly less than 100 ng of high-quality DNA, consult section I. Library Amplification Optimization with qPCR.

2. Spin down P3 and place on ice.
3. Thaw P2 at room temperature. Mix by vortexing, spin down and place on ice.
4. Prepare a master mix by combining P2 and P3 in a 0.5 mL capped tube according to the volumes shown in Table 10. Mix well by pipetting, taking care to avoid bubbles, spin down and place on ice.
5. On ice, add 30 µL of Amplification Master Mix to each sample for a total of 50 µL.
6. Place tubes in a pre-warmed thermal cycler programmed to run Program 6 (Library Amplification; see Table 3):

   95 °C – 2 min, N(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s), hold at 10 °C
7. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

Optional stopping point: Store samples at –20 °C.

K. Amplified Library Purification

1. Ensure the Agencourt beads and DR1 have completely reached room temperature before proceeding.
2. Resuspend the beads by inverting and tapping the tube. Ensure the beads are fully resuspended before adding to samples. After resuspending, do not spin the beads.
3. Add 50 µL (1 volume) of the bead suspension to each reaction. Mix thoroughly by pipetting 10 times.
4. Incubate at room temperature for 10 minutes.
5. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
6. Carefully remove 90 µL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

V. Protocol

Note: The beads should not disperse; instead, they will stay on the walls of the tubes. Significant loss of beads at this stage will impact the final yield, so ensure beads are not removed with the binding buffer or the wash.

7. With samples still on the magnet, add 200 µL of freshly prepared 70% ethanol.
8. Remove the 70% ethanol wash using a pipette.
9. Repeat steps 7 and 8 for a total of two washes.

Note: With the final wash, it is critical to remove as much of the ethanol as possible. Use at least two pipetting steps and allow excess ethanol to collect at the bottom of the tubes after removing most of the ethanol in the first pipetting step.

10. Air dry the beads on the magnet for 10 minutes. Inspect each tube carefully to ensure that all of the ethanol has evaporated. It is critical that all residual ethanol be removed prior to continuing.
11. Remove the tubes from the magnet.
12. Add 20 µL DR1 to the dried beads. Mix thoroughly to ensure all beads are resuspended.
13. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.
14. Carefully remove 18 µL of the eluate, ensuring as few beads as possible are carried over and transfer to a fresh set of PCR tubes.

Optional stopping point: Store samples at –20 °C.

L. Quantitative and Qualitative Assessment of the Library

1. Measure library concentration using 2 µL of each library with the Qubit® Fluorometer and dsDNA HS Assay Kit (Life Technologies). Dilute an aliquot to 5 ng/µL using DR1 buffer and load 1 µL on the Bioanalyzer High Sensitivity DNA Chip. Fragment distribution should be as shown in **Figure 9**. The three peaks at 200 bp, 265 bp, and 330 bp are due to MspI-containing micro-satellite repeats, and are characteristic of RRBS libraries made from human DNA.
2. Quantify the library using a qPCR-based method. Use 250 bp as the library size for calculations.

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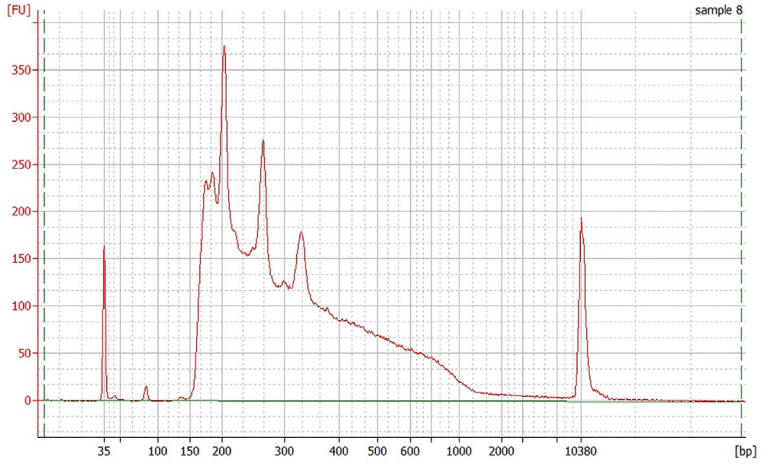
Figure 9. Fragment distribution when 1 µL of 5 ng/µL library is loaded into a High Sensitivity DNA Chip from 100 ng human DNA.



3. Normalize and pool libraries following the Illumina guidelines "Best practices for manually normalizing library concentrations" and the "Low-Diversity Sequencing" guidelines for your specific sequencer. See Appendix A. of this guide for guidelines on color balancing and multiplexing of Tecan libraries.

VI. Technical Support

For help with any of our products, please contact Tecan NGS Technical Support at 650.590.3674 (direct) or 888.654.6544, option 2 (toll-free, U.S. only). You may also send faxes to 888.296.6544 (toll-free) or email techserv-gn@tecan.com.

In Europe contact Tecan NGS Technical Support at +31(0)135780215 (Phone) or +31(0)135780216 (Fax) or email europe-gn@tecan.com.

In all other locations, contact your Tecan distributor for technical support.

VII. Appendix

A. Barcode Sequences and Guidelines for Multiplex Experiments

Barcode sequences and multiplex guidelines for adaptors used in Ovation RRBS Methyl-Seq System 1–16 can be found in Table 11. These 6-nucleotide barcode sequences must be input into the Illumina Sequencing System prior to parsing of the data.

You may combine anywhere from 2–16 barcoded libraries to allow for a range of multiplex sequencing. However, the barcodes were carefully chosen for their ability to parse properly and for color balancing and therefore have strict pairing requirements when performing 2-plex multiplexing. Users wishing to perform greater than a 2-plex multiplexing must choose a Duplex Set (as defined in Table 11), combined with any of the remaining barcoded libraries.

All barcode sequences are separated by an edit distance of three. For further details on the barcode design strategy, please refer to Faircloth BC, Glenn TC (2012) Not All Sequence Tags Are Created Equal: Designing and Validating Sequence Identification Tags Robust to Indels. *PLoS ONE* 7(8): e42543. doi:10.1371/journal.pone.0042543.

If you choose to mix Ovation RRBS Methyl-Seq System libraries with other libraries, check to ensure that the barcodes are compatible (i.e. can be parsed). If you intend to parse with one mismatch allowed, make sure that the spike in library barcodes are an edit distance of 3 or greater from the RRBS barcodes used in that lane. The PhiX library from Illumina does not contain an index. As a result, the sequencer will produce a low-quality index read from PhiX clusters. To remove PhiX reads prior to parsing, filter by index read quality and remove reads with quality less than 20.

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Table 11. Barcode sequences for Ovation RRBS Methyl-Seq adaptors.

LIGATION ADAPTOR MIX	BARCODE SEQUENCE	BARCODE PAIRING (2-PLEX)	BARCODE PAIRING (> 2-PLEX)
L2V20DR-BC1	AACCAG	Duplex Set 1	One of the duplex sets from the column to the left must be used in combination with any of the other remaining 14 individual barcodes.
L2V20DR-BC2	TGGTGA		
L2V20DR-BC3	AGTGAG	Duplex Set 2	
L2V20DR-BC4	GCACTA		
L2V20DR-BC5	ACCTCA	Duplex Set 3	
L2V20DR-BC6	GTGCTT		
L2V20DR-BC7	AAGCCT	Duplex Set 4	
L2V20DR-BC8	GTCGTA		
L2V20DR-BC9	AAGAGG	Duplex Set 5	
L2V20DR-BC10	GGAGAA*		
L2V20DR-BC11	AGCATG	Duplex Set 6	
L2V20DR-BC12	GAGTCA		
L2V20DR-BC13	CGTAGA	Duplex Set 7	
L2V20DR-BC14	TCAGAG		
L2V20DR-BC15	CACAGT	Duplex Set 8	
L2V20DR-BC16	TTGGCA		

** This barcode starts with GG and therefore may not be compatible with low-plex pooling on two-color sequencing platforms (e.g. NextSeq). Please refer to the latest recommendations for multiplexing on your specific sequencer.*

B. Working with Low Input and Degraded Samples

In the standard Ovation RRBS Library System protocol, libraries are generated using 100 ng of high-quality, MspI-digested genomic DNA. Although not recommended, it is possible to generate RRBS libraries from less than 100 ng of gDNA, or from degraded gDNA, such as DNA extracted from formalin fixed, paraffin embedded (FFPE) specimens. Library Amplification will require additional cycles of PCR.

You may observe an increase in adaptor artifacts, as well as decreased library complexity, in libraries made from less than 100 ng or degraded samples that require additional cycles of PCR amplification. Adaptor artifacts typically appear as a peak of approximately 145 bp. These artifacts can be reduced by performing a second bead purification. Add 1 volume of beads to the purified library, and follow the amplified library purification protocol.

Signs of reduced complexity should be monitored by sequencing the unique 6 random bases appended to the index on each reverse adaptor molecule, then using the Duplicate Marking tool (NuDup; see

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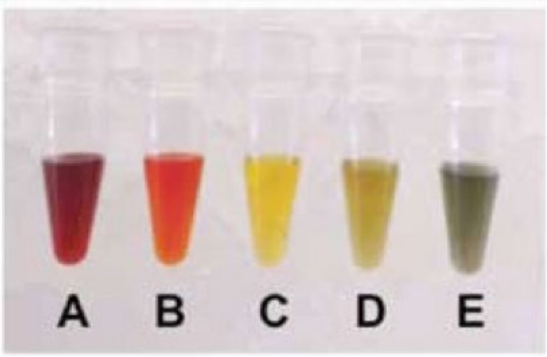
Appendix D) to identify unique molecules. After removing true PCR duplicates you will have a reliable measure of library complexity. Therefore, sequencing the additional 6 bases after the 6-base barcode is especially useful when attempting RRBS on rare or degraded samples. See Section III. E. and III. F. for information on index structure and sequencing, and Appendix D for information on duplicate marking and removal.

C. Oxidant Color Changes

Upon receipt of the kit, the color of the thawed Oxidant Solution should resemble Solution A in **Figure 10**. If the Oxidant Solution looks dark yellow or green with considerable amounts of black precipitate (Solution D or E), it is a sign that the reagent has become exposed to contaminants or CO_2. If you suspect that this has occurred, please do not use the Oxidant Solution and contact Tecan NGS technical support for advice.

Figure 10. Expected Oxidant Solution Color Changes



A. Oxidant Solution stock concentration.
B. 10-fold dilution of the Oxidant Solution in alkaline solution.
C. Working oxidant concentration (WOC).
D. 5:1 molar excess of WOC:ethanol.
E. 20:1 molar excess of ethanol:WOC.

During oxidation (section V. F.), the color of the oxidation reaction should be similar to Solution B or C in **Figure 10**. It is normal for a small amount of black precipitate to form during the oxidation reaction. The purpose of the strong centrifugation step following oxidation is to pellet the dark precipitate and enable removal of the clear orange/yellow solution without this contaminant. After the oxidation reaction and subsequent 10 minute centrifugation are complete, the solution should remain orange indicating a successful oxidation.

If the post-oxidation color appears as Solution D, it suggests partial decomposition of the oxidant, indicating that oxidation of the DNA samples was successful. However, if the post-oxidation color resembles Solution E, significant decomposition of the oxidant has likely occurred, resulting in incomplete conversion of 5-hmC --> U. In this instance, it is recommended to re-purify the sample in order to remove contaminants from the starting DNA sample solution. To avoid contaminants, ensure all guidance regard-

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ing oxidation solution sensitivity in section III. C. is followed, including the use of only Ultra Pure Water provided with the TrueMethyl oxBS Module in steps containing the oxidant.

As a final note on color changes, samples that have been processed using the Oxidant Solution may also take on a light grey color after addition of the Bisulfite Conversion Solution (section V. G.). This is normal and will not impact downstream processing.

D. Data Analysis of Ovation RRBS Methyl-Seq Libraries

Ovation RRBS Methyl-Seq libraries feature a diversity sequence that eliminates the need for PhiX during sequencing. Following sequencing and parsing you will need to trim adaptors and remove the additional sequence added by the diversity adaptors to the 5′ and 3′ ends of the insert before downstream analysis. Data analysis tools and recommendations can be found using the link below.

https://github.com/nugentechnologies/NuMetRRBS

E. Frequently Asked Questions (FAQs)

Getting Started

Q1. Does Tecan provide reagents for performing the bisulfite conversion step of the protocol?
The bisulfite conversion reagents are included with the purchase of product part numbers 0553-32. This bundle includes the Ovation RRBS Methyl-Seq core kit (Part No. 0353) and the TrueMethyl oxBS module (Part No. 0414).

Input Recommendations

Q2. Can I use less than 100 ng of input DNA into the Ovation RRBS Methyl-Seq System?
The Ovation RRBS Methyl-Seq system with the TrueMethyl oxBS workflow has not been tested for inputs less than 100 ng. Although not recommended, the Ovation RRBS Methyl-Seq System core kit may be used with third-party bisulfite conversion kits for less than 100 ng inputs. See Appendix B. for recommendations on working with low-input samples.

General Workflow

Q3. Can I prepare extra Desulfonation Buffer? Can I save leftover Desulfonation Buffer for future use?
While we recommend preparing the buffer fresh with each use, extra/leftover buffer can be stored at 4 °C. To prevent ethanol evaporation, tightly seal the cap and wrap with parafilm.

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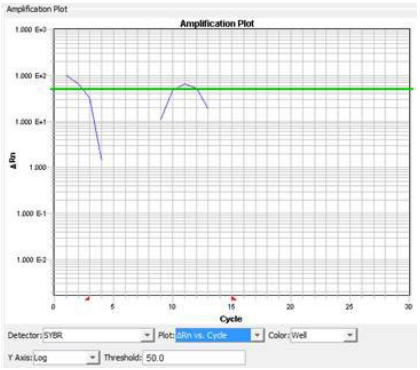
Q4. Can I use restriction enzymes other than, or in addition to, MspI?
Yes, however the restriction site must leave a similar 5'-CG overhang in order for the ligation to be effective. Please contact Tecan NGS technical support for further advice on integrating other enzymes into this protocol.

Q5. Can I prepare MBBS1 and MBBS2 in advance/prepare extra solution?
We recommend always preparing MBBS1 and MBBS2 fresh on the day of use. If reagent is prepared in advance or if excess reagent is prepared, store at 4 °C and use within one week. Discard after 1 week.

Q6. Can I combine the barcoded libraries prior to amplification?
The stoichiometry of barcoded libraries may be adversely affected by this modification to the workflow. We suggest that the libraries be amplified and quantitated independently before being balanced and pooled for use on the sequencer.

Q7. Why doesn't my Library Amplification qPCR plot resemble the example in the user guide?
Certain real-time PCR instruments may display unexpected results, such as the example in **Figure 11**. Ensure that your plot is set to display Rn. vs. Cycle, not deltaRn vs. Cycle, and that the y-axis is set to a log scale.

Figure 11. Results from selecting Plot > deltaRn vs. Cycle.



SPRI Bead Purifications

Q8. What is the difference between RNAClean XP and AMPure XP SPRI beads? Can both be used interchangeably?
RNAClean XP beads are certified to be RNase and DNase free. We have tested both RNAClean XP and AMPure XP beads in our kits and observe no difference in performance between products.

Q9. What magnetic separation devices do you recommend for the SPRI bead purifications?
Due to the large number of commercially available magnets, we do not have a comprehensive list of compatible products. However, many magnets are compatible. As long as the magnet is strong enough to clear the solution of magnetic beads, it can be applied to the system. We have the following guidelines for selecting a magnetic separation device:

- Use a magnet designed for 0.2 mL tubes (PCR tubes), tube strips, or plates. Compared to magnets that are designed for 1.5 mL tubes, these minimize loss that can occur when samples are transferred from one tube to another.
- Prior to purchasing, check the manufacturer's specifications for minimum and maximum volumes that can be effectively treated.

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Test the magnet with a mock purification to ensure the magnet will effectively clear the solution under the conditions in the Tecan workflow. This is also helpful to gain familiarity with the purification workflow.

Q10. How can I ensure maximum recovery of sample from the SPRI bead purification?

- Allow the SPRI beads to reach room temperature before use; cold beads result in lower yields.
- Ensure that the beads are fully resuspended in solution before adding to the sample.
- Always use fresh ethanol during the washing steps. When preparing the ethanol, measure out the ethanol and water separately to ensure the desired ethanol concentration is obtained.
- Mix the bead suspension and sample thoroughly to ensure maximum binding of the samples to the beads.

Library Quantification/Qualification

Q11. How do I measure my final library yield? Can I use an Agilent Bioanalyzer to evaluate the product?

Please refer to section V. L. for guidelines on quantitative and qualitative assessment. We recommend using a qPCR based-method in combination with the Agilent Bioanalyzer or Tapestation for the most accurate quantification.

Q12. What is the expected yield of the amplified DNA library using the Ovation RRBS Methyl-Seq System 1–16?

The expected yield is at least 200 ng, depending on the quality and quantity of the genomic DNA and the number of PCR cycles employed. This amount is in excess of the amount of DNA required for cluster generation.

Q13. How many bases do the Ovation RRBS Methyl-Seq System adaptors add to the library?

The adaptors add 145 bp to the library.

Sequencing Recommendations

Q14. What kind of sequencing primers can I use with your library?

The design of the Ovation RRBS Methyl-Seq System 1–16 requires the use of a custom Read 1 sequencing primer, MetSeq Primer 1, which is included in this kit at a concentration of 100 µM. The standard primers provided in the Illumina sequencing kit are sufficient for Read 2 and for sequencing the barcodes (Index Read). The Standard Read 1 Primer is also required when using PhiX or other libraries to increase base complexity. The Standard Read 1 Primer should be mixed with MetSeq Primer 1 for sequencing of these libraries with PhiX.

Q15. Can the Ovation RRBS Methyl-Seq System 1–16 be used with paired-end sequencing?

Yes, it can be used for both single end and paired-end sequencing.

Q16. How much material should I load into the sequencer?

Please follow manufacturer's recommendations for library QC, quantitation, balancing and loading of the amplified library on the sequencer.

Q17. Is the Ovation RRBS Methyl-Seq System compatible with all Illumina sequencing platforms?

Illumina may not support the use of a custom sequencing primer or low diversity libraries on all platforms. Please follow the custom primer and low-diversity library recommendations for your specific sequencer.

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Q18. How can I identify PCR duplicates in my Ovation RRBS Methyl-Seq libraries?
Ovation RRBS Methyl-Seq libraries feature a 6 bp molecular tag after the 6 bp barcode. To take advantage of this feature, libraries must be sequenced with a 12 bp index read following the instructions in Section III. E and analyzed using the instructions in Appendix D.

Q19. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?
Each of the DR barcode sequences shown in Table 11 are separated by an edit distance of three. This means that three events, such as insertion, deletion or substitution must occur before any barcode sequence is converted into another barcode sequence. A benefit of having an edit distance of three in the barcodes is that one error can be corrected without the chance of barcode misassignment. Parsing software can be adjusted to perform such error correction if there is a large proportion of unsegregated reads.

Q20. Are the libraries directional?
Yes, the libraries are directional due to the way our library system is designed and the nature of bisulfite conversion. The forward sequencing reads will correspond to a bisulfite-converted version of either the original top or the original bottom strand (the C-to-T reads) and the reverse sequencing reads will correspond to the complement of the original top or the complement of the original bottom strand (the G-to-A reads). In contrast, a non-directional bisulfite converted library will have all four possible strands in the forward read (original top, original bottom, complement of original top and complement of original bottom).

Q21. What analysis software can be used for aligning, methylation calling, and visualization of your bisulfite sequencing data?
The number of analysis strategies and software tools for methylation-based sequencing studies is growing rapidly. The ideal analysis workflow for a given experiment depends on many variables, including the type of experiment and the goals of the study. Currently, Tecan scientists use Bismark for aligning and determining methylation status. This program utilizes the Bowtie aligner (www.bioinformatics.bbsrc.ac.uk/projects/bismark/). The Broad IGV genome browser can be used to visualize the results of Bismark (www.broadinstitute.org/igv/). Data analysis recommendations can be found here: https://github.com/nugentechnologies/NuMetRRBS

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Q22. How can I measure the efficiency of bisulfite conversion?

DNA material that is known to be unmethylated, such as lambda DNA, can be used to measure the efficiency of C-to-U conversion in the bisulfite conversion kit. This control DNA is not included with the Ovation RRBS Methyl-Seq System 1–16.

F. Update History

This document, the Ovation RRBS Methyl-Seq System 1–16 user guide (M01394 v7), has been updated from the previous version to address the following topics:

Description	Section	Page(s)
TrueMethyl oxBS Module updates made throughout.	Throughout	Throughout
Changed NuGEN/NuGEN Technologies to Tecan/Tecan Genomics.	Throughout	Throughout
Minor changes and corrections made throughout.	Throughout	Throughout

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