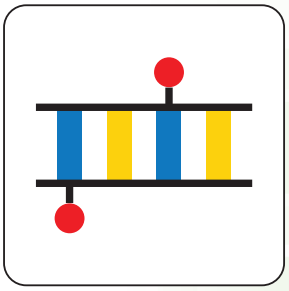
USER GUIDE



Ultralow Methyl-Seq with TrueMethyl® oxBS

Catalog Numbers: 0541, 9513

Publication Number: M01512

Revision: v2

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I. Introduction

A. Background

The Ultralow Methyl-Seq with TrueMethyl oxBS (Ultralow Methyl-Seq) provides a simple, fast and scalable solution for producing directional bisulfite-converted libraries for next generation sequencing, from 10-300 ng of high quality genomic DNA. The library construction workflow is designed specifically for whole genome bisulfite sequencing on Illumina NGS platforms.

The Ultralow Methyl-Seq kit and is compatible with Illumina sequencing platforms. This kit is available with 32 and 96 (Part Nos. 0541 and 9513, respectively) unique barcoded adaptors for multiplex sequencing. Each well contains sufficient volume for preparation of a single library.

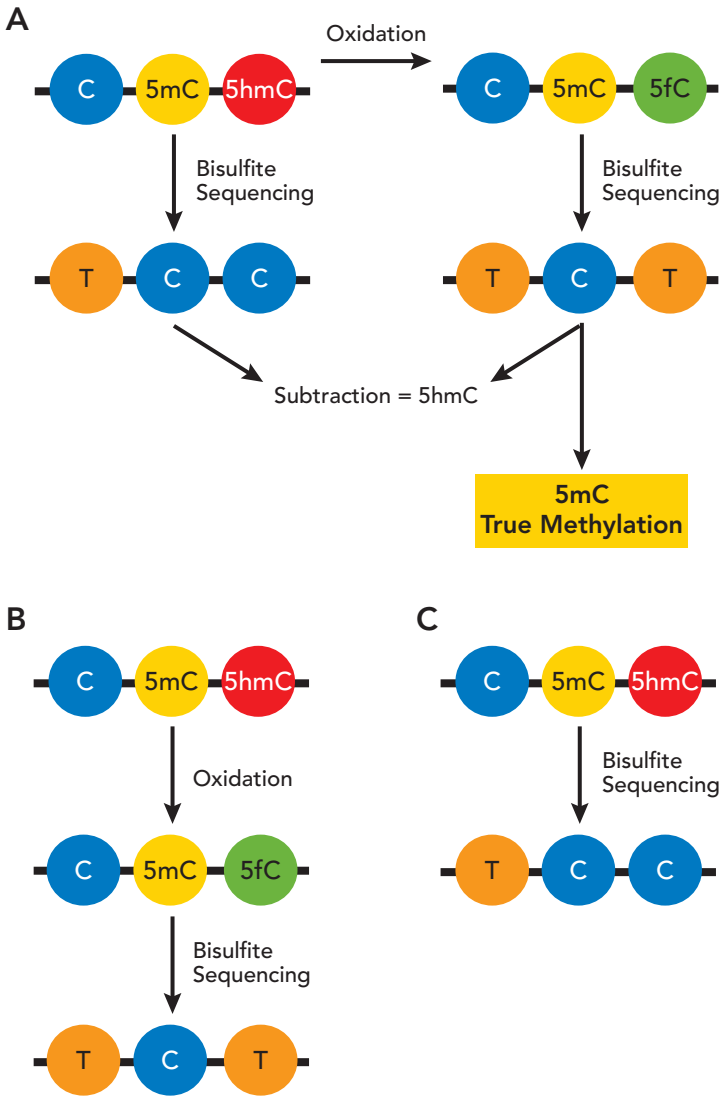
TrueMethyl oxBS Module

The Ultralow Methyl-Seq kit includes the TrueMethyl oxBS Module (Part No. 0414), which allows for flexible methyl-seq workflows. In conjunction with the Ultralow Methyl-Seq kit, the TrueMethyl oxBS Module enables interrogation of both 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC) for inputs of 100-300 ng genomic DNA, providing a method to accurately quantify the true level of 5mC. 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)).

I. Introduction

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 1A**). For those interested only in 5mC, there are sufficient reagents to process 32 individual samples through the oxBS workflow (**Figure 1B**). Alternatively, 32 samples may be processed through the bisulfite-only workflow for indiscriminate detection of 5mC and 5hmC (**Figure 1C**).

Figure 1. TrueMethyl oxBS Module workflow options.



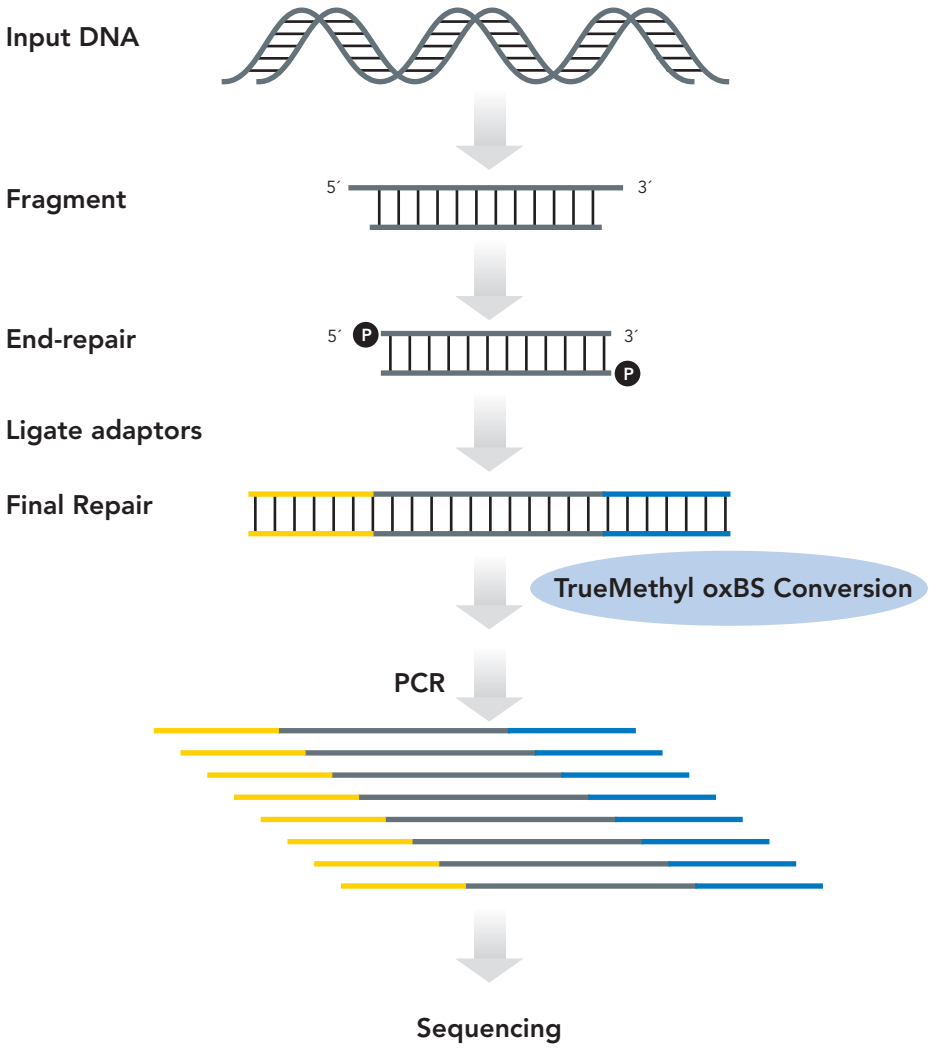
The TrueMethyl oxBS Module (Part No. 0414) is fully compatible and is included with the Ultralow Methyl-Seq kit (0541-32, 9513-A01).

I. Introduction

B. Workflow

As shown in **Figure 2**, the streamlined workflow consists of five main steps: fragmentation of genomic DNA, end repair to generate blunt ends, adaptor ligation, optional oxidation, bisulfite conversion, and PCR amplification to produce the final library. With the TrueMethyl oxBS Module, parallel workflows with and without oxidation can be performed for analysis of 5hmC. The entire workflow, including fragmentation, can be completed in less than two days and generates DNA libraries ready for single read or paired-end sequencing.

Figure 2. Schematic of the Ultralow Methyl-Seq with TrueMethyl oxBS workflow.



I. Introduction

C. Perfomance Specifications

The Ultralow Methyl-Seq with TrueMethyl oxBS kit is designed to produce DNA libraries suitable for either single read or paired-end sequencing on Illumina NGS platforms using as little as 10 ng of high quality genomic DNA as input. This system generates sequencing-ready libraries in less than two days.

D. Quality Control

Every lot of the Ultralow Methyl-Seq with TrueMethyl oxBS kit 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.

Ultralow Methyl-Seq core kit (Part No. 0366 and 9113)

The Ultralow Methyl-Seq kit 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.

All other components 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 (Part No. 0414)

The TrueMethyl oxBS Module is shipped in two separate boxes and separately from the Ultralow Methyl-Seq library prep kit.

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 Tecan website at www.nugen.com/products/ultralow-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 Ultralow Methyl-Seq with TrueMethyl oxBS page at NuGEN.com (www.nugen.com/products/ultralow-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. Ultralow Methyl-Seq core kit reagents (Part No. 0366, 9113)

COMPONENT	PART NUMBER (0366-32)	PART NUMBER (9113-A01)	VIAL LABEL	VIAL NUMBER
End Repair Buffer Mix	S01758	S02163	Blue	ER1 VER 8
End Repair Enzyme Mix	S01533	S02164	Blue	ER2 VER 4
End Repair Enhancer	S01709	S02165	Blue	ER3 VER 2
Ligation Buffer Mix	S01534	S01689	Yellow	L1 VER 4
Ligation Enzyme Mix	S01535	S01690	Yellow	L3 VER 4
Final Repair Buffer Mix	S01759	S02166	Purple	FR1 VER 4
Final Repair Enzyme Mix	S01569	S01696	Purple	FR2
Amplification Primer Mix	S01739	S02172	Red	P2 VER 8
Amplification Enzyme Mix	S01740	S02167	Red	P3 VER 3
100 µM Sequencing Primer	S02670	S02671	Orange	MetSeq Primer 1
Nuclease-free Water	S01001	S01001	Green	D1
32-Plex Adaptor Plate	S02694	—	Yellow	—
96-Plex Adaptor Plate	—	S02169	Yellow	L2V11DR-BC 1-96
Agencourt Beads	S01502	—	Clear	—

II. Components

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

DESCRIPTION	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

- **Equipment**
  - Covaris S-series Sonication System
  - 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, heated lid, and 100 µL reaction capacity
  - Heat block, thermomixer or heated orbital incubator able to maintain temperatures at 37 °C and 60 °C (e.g. Eppendorf® Thermomixer Comfort), for the 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
  - EvaGreen® Dye, 20X in water (Biotium, Cat. #31000)
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793), optional; for diluting nucleic acids

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 Tecan
  - 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 Ultralow Methyl-Seq kit is designed to work with inputs up to 300 ng of fragmented genomic DNA. For large genomes, such as human, inputs of less than 50 ng may result in lower complexity libraries. For smaller genomes, libraries can successfully be made from inputs lower than 50 ng.

Workflow	Minimum Input, Complex Genomes	Minimum Input, Small Genomes
Bisulfite only	50 ng	10 ng
Oxidation + Bisulfite	100 ng	100 ng

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 and the A260:A230 ratio in excess of 2.0. Using DNA samples with lower ratios may compromise your results.

DNA Integrity

Although not recommended, it is possible to generate libraries from less than 10 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 on using challenging samples and low inputs.

B. Working with the 32- or 96-Plex Adaptor Plate

The Adaptor Plate included with the 32 and 96 reaction Ultralow Methyl-Seq kits contain adaptor mixes, each with a unique eight-base barcode. Each well (first 32 wells, A01– H04, or all 96 wells, respectively) contains sufficient volume for preparation of a single library.

Ultralow Methyl-Seq Adaptor Plates are sealed with a foil seal designed to provide airtight storage. Thaw adaptor plate on ice and spin down. Do NOT warm above room temperature. Make sure all adaptor mixes are collected at the bottom of the wells and place the adaptor plate on ice after centrifuging. When pipetting the adaptor mixes, puncture the seal for each well you wish to use with a fresh pipet tip, and transfer the appropriate volume of adaptor into your sample. The remaining wells of the plate should remain sealed for use at a later date. Cover used wells with a new foil seal (e.g., AlumaSeal II) to prevent any remaining adaptor-containing liquid from contaminating future reactions.

For details regarding barcode color balancing for multiplex sequencing, please see Appendix A on page 33.

III. Planning the Experiment

C. 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 vapour 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.

D. Oxidant Solution Sensitivity

Important: The reactivity of this reagent allows the selective oxidation of 5hmC to 5fC. However, the oxidant will react with other contaminating compounds 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 as well as 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.

III. Planning the Experiment

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 in a -80 °C freezer.

E. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

F. Using Ultralow Methyl-Seq on Illumina NGS Platforms

Libraries generated with the Ultralow Methyl-Seq kit are compatible with Illumina NGS platforms. These libraries should be sequenced using the Illumina protocol for multiplex sequencing. The barcode sequences are found in Appendix A.

Important:

- The design of the Ultralow Methyl-Seq kit 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. Sequencing with custom primers may require different considerations and configurations on different Illumina platforms. Please follow the custom primer recommendations for your specific sequencer.
- The standard Illumina Read 1 Primer is required to sequence PhiX, as well as when multiplexing with other libraries. MetSeq Primer 1 should be mixed with the Standard Read 1 primer to sequence PhiX and/or other libraries. Follow the Illumina guidelines for the proper percentage of PhiX to include in the sequencing run, as it may differ from platform to platform. 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 on four color systems. Therefore, strict requirements exist for choosing barcoded libraries for a multiplexed sequencing run. Refer to Appendix A for multiplexing guidelines.

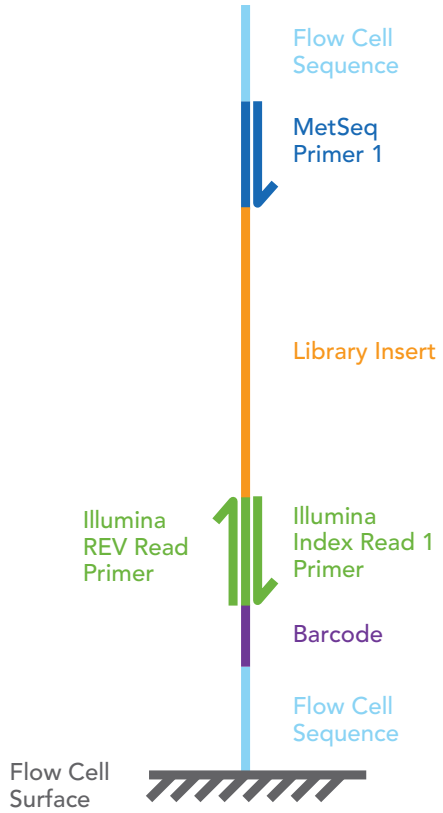
The Ultralow Methyl-Seq kit produces directional bisulfite-converted libraries. This means Read 1 will sequence the C-to-T converted strand and the overall nucleotide balance for Read 1 will show a low proportion of C bases.

III. Planning the Experiment

Illumina has recommendations for how to obtain high quality base calls from libraries containing unbalanced nucleotide ratios. Please refer to the Illumina technical support for your specific instrument for more information.

G. Data Analysis and Parsing Multiplex Libraries

Figure 3. Ultralow Methyl-Seq Library Structure



The Ultralow Methyl-Seq kit uses the same approach to multiplexing as the standard Illumina method and should be sequenced using the Illumina protocol for multiplex sequencing. Follow the recommendations in the Illumina technical support documentation on parsing barcodes, using the Ultralow Methyl-Seq barcode sequences provided in Appendix A. Note that a slightly higher rate of unmatched barcodes may be observed with bisulfite-converted libraries relative to non-bisulfite-converted libraries.

Once the data have been parsed according to sample, additional sample-specific data analysis may be employed according to the requirements of the experiment. Please see Appendix C for detailed recommendations on trimming and alignment of bisulfite- and oxidative bisulfite-converted libraries.

IV. Overview

A. Overview

The library preparation process used in the Ultralow Methyl-Seq kit is performed in the following stages:

		TrueMethyl oxBS Workflow
1.	End repair	0.75 hours
2.	Adaptor ligation and purification	1.25 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 library	**9.25 hours**

Components in the Ultralow Methyl-Seq kit 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 sequencing. 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 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 steps. These steps 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 samples 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.

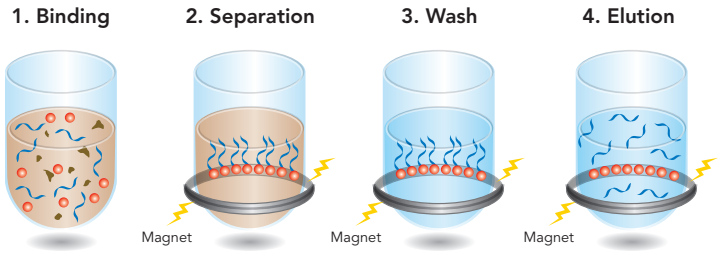
C. Magnetic Beads

Magnetic beads (Agencourt Beads and Magnetic Bead Solution) are provided for use with the Ultralow Methyl-Seq library prep kit and TrueMethyl oxBS Module, respectively. 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 4. 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, 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. For thermal cyclers with a fixed-temperature heated lid use the default settings (typically 100 to 105 °C).

Table 3. Thermal Cycler Programming

END REPAIR		VOLUME
Program 1 End Repair	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	16 µL
ADAPTOR LIGATION		**VOLUME**
Program 2 Ligation	25 °C – 30 min, 70 °C – 10 min, hold at 4 °C	31 µL
FINAL REPAIR		**VOLUME**
Program 3 Final Repair	60 °C – 10 min, hold at 4 °C	40 µL
OXIDATION		**VOLUME**
Program 4 Denaturation	37 °C – 5 min, hold at 25 °C	10 µL
BISULFITE CONVERSION		**VOLUME**
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		**VOLUME**
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.

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.

- Two independent NGS libraries should be prepared for each genomic DNA sample to be analyzed for 5hmC. Process one aliquot (100 ng-300 ng) through the oxBS workflow and the other aliquot (100 ng-300 ng) through the parallel MOCK oxBS workflow. The MOCK oxBS workflow excludes the oxidant solution. We recommend creating the aliquots after Covaris fragmentation and purification.
- 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.
- Bisulfite conversion is required with this kit. For alternate workflows without the TrueMethyl oxBS Module, substitute the third party bisulfite treatment for protocol sections V.H. - V.K. Step V.M. Library Amplification is designed to accommodate a volume of 20 µL of bisulfite converted, desulfonated DNA.

1. Aliquot each input sample (10-300 ng) into a 0.2 mL or 0.5 mL tube.
2. Adjust each sample to 50 µL with 1X TE buffer (low EDTA).

B. DNA Fragmentation

We recommend fragmentation using the Covaris Adaptive Focused Acoustics method, following the manufacturer's recommendations for the desired fragment length.

Fragment length should be chosen with consideration for the desired sequencing read length, and whether the sequencing will be single-end or paired-end.

Fragmentation should generate a uniform distribution of library molecules for optimal library preparation. Other means of fragmentation may be also be suitable, but have not been tested by Tecan and will require validation. For alternatives to the Covaris system, please contact Tecan NGS Technical Support. The protocol below, for fragmenting DNA to ~200 bp using a Covaris S-series instrument, has been successfully used at Tecan and is provided for your convenience.

Note: Prior to fragmentation, remove the Agencourt beads from 4 °C and place at room temperature.

1. Transfer 50 µL of each sample from step 2 above to a Covaris snap-cap microTUBE.
2. Fragment to a 200 bp length using the Covaris settings listed in Table 5. For other fragment sizes, follow specifications as provided by the manufacturer.

V. Protocol

3. After fragmentation, briefly spin the Covaris tube to collect any remaining liquid from the sides of the tube. Using a pipette, pierce the rubber septum with the tip and transfer the entire sample to a new set of PCR tubes or plates.

Table 4. Covaris S-Series System Settings

PARAMETER	VALUE
Target BP	200
Intensity	5
Duty Cycle	10%
Cycles/burst	200
Treatment Time	2 x 1 minute (2 minutes total)
Water Bath Temperature	7 °C
Water Level-S2	12
Sample Volume	50 µL

C. Fragmented DNA Purification

Choose a nucleic acid column-based purification system that allows small volume elution, such as the MinElute® Reaction Cleanup Kit (QIAGEN, Cat. #28204). Alternatively, you may use the Agencourt bead-based purification protocol detailed below, which is provided for your convenience. This protocol is designed to work with a starting volume of 50 µL of fragmented DNA. If your starting volume is different, change the volume of beads added in Step 5 to 1.8 times the volume of your fragmented DNA.

1. Ensure the Agencourt beads and Nuclease-free Water (D1) have completely reached room temperature before proceeding.
2. Resuspend beads by vortexing the tube. Ensure beads are fully resuspended before adding to sample. After resuspending, do not spin the beads.
3. Prepare a fresh 70% ethanol wash solution.
4. Briefly spin down samples.
5. Add 90 µL (1.8 volumes) of bead suspension to each reaction. Mix thoroughly by pipetting 10 times.
6. Incubate at room temperature for 10 minutes.
7. Transfer the tubes to the magnet and let stand 5 minutes to completely clear the solution of beads.
8. Carefully remove the binding buffer and discard it.

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 amount of purified DNA, so ensure beads are not removed with the binding buffer or the wash.

V. Protocol

9. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
10. Remove the 70% ethanol wash using a pipette.
11. Repeat steps 9 and 10 for a total of two 70% ethanol 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.

12. Air dry the beads on the magnet for 5–10 minutes. Inspect each tube carefully to ensure that all the ethanol has evaporated.
13. Remove the tubes from the magnet.
14. Add 14 µL room temperature of 1X low-EDTA TE buffer or Nuclease-free Water (green: D1) to the dried beads. Mix thoroughly to ensure all the beads are resuspended.
15. Transfer the tubes to the magnet and let stand for 5 minutes to completely clear the solution of beads.
16. Carefully remove 13 µL of the eluate, ensuring as few beads as possible are carried over; transfer to a fresh set of PCR tubes and place on ice.

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

D. End Repair

Table 5. End Repair Master Mix

REAGENT	END REPAIR BUFFER MIX (BLUE: ER1 VER 8)	END REPAIR ENZYME MIX (BLUE: ER2 VER 4)	END REPAIR ENHANCER (BLUE: ER3 VER 2)
STORAGE	–20 °C	–20 °C	–20 °C
1X REACTION VOLUME	2.0 µL	0.5 µL	0.5 µL

1. Thaw ER1 at room temperature. Mix by vortexing, spin down and place on ice.
2. Spin down ER2 and ER3 and place on ice.
3. Obtain 13 µL of fragmented DNA sample (10–300 ng) from the DNA Purification protocol.

Note: To interrogate 5hmC, split the sample into two 13 µL aliquots to perform parallel library preparations with oxBS and MOCK oxBS workflows. The aliquots can be diluted using Nuclease-free Water or 1X low-EDTA TE buffer.

4. Prepare a master mix by combining ER1, ER2 and ER3 in a 0.5 mL capped tube, according to the volumes shown in Table 5.
5. Add 3 µL of End Repair Master Mix to each sample tube for a total of 16 µL.

V. Protocol

6. Mix by pipetting, cap and spin tubes and place on ice.
7. Place tubes in a pre-warmed thermal cycler programmed to run Program 1 (End Repair; see Table 3):

   25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
8. Remove tubes from the thermal cycler, spin to collect condensation and place on ice.

E. Adaptor Ligation

1. Remove Agencourt beads from 4 °C. Place on the bench top to reach room temperature for use in the next step.

Table 6. Ligation Master Mix

REAGENT	NUCLEASE-FREE WATER (GREEN: D1)	LIGATION BUFFER MIX (YELLOW: L1 VER 4)	LIGATION ENZYME MIX (YELLOW: L3 VER 4)
STORAGE	---	-20 °C	-20 °C
1X REACTION VOLUME	4.5 µL	6 µL	1.5µL

2. Spin down L3 and place on ice.
3. Thaw adaptor plate on ice, spin down, and return to ice.
4. Thaw L1 at room temperature. Mix by vortexing, spin down and place on ice.
5. Add adaptors to each sample as follows:
   a. If using adaptors from 32-plex Adaptor Plate (S02694), add 3 µL of the appropriate Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting.
   b. If using adaptors from 96-plex Adaptor Plate (S02169), add the entire 16 µL of sample to the appropriate adaptor well, mix well by pipetting, then transfer the entire sample to a PCR tube.

Note: In order to multiplex the oxBS and MOCK oxBS processed samples, unique indexes are required.

6. Prepare a master mix by combining D1, L1 and L3 in a 0.5 mL capped tube, according to the volumes shown in Table 6. Mix by pipetting slowly, without introducing bubbles, spin and place on ice. Use the master mix immediately.

Note: The L1 Ligation Buffer Mix is very viscous. Pipet this reagent slowly and mix thoroughly.

7. Add 12 µL Ligation Master Mix to each reaction for a total of 31 µL. Mix thoroughly by pipetting slowly and gently, spin down and place on ice. Proceed immediately with the incubation.
8. Place 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
9. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

V. Protocol

F. Post-Ligation Purification

1. Ensure the Agencourt beads and D1 have completely reached room temperature before proceeding.
2. Resuspend the beads by vortexing the tube. Ensure the beads are fully resuspended before adding to the sample. After resuspending, do not spin the beads.
3. At room temperature, add 45 µL (1.5 volumes) 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 the binding buffer and discard it.

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 amount of purified DNA, so ensure beads are not removed with the binding buffer or the wash.

7. With the tubes still on the magnet, add 200 µL of freshly prepared 70% ethanol and allow to stand for 30 seconds.
8. Remove the 70% ethanol wash using a pipette.
9. Repeat steps 7 and 8 for a total of two 70% ethanol 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 5–10 minutes. Inspect each tube carefully to ensure that all 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 16 µL room temperature of 1X low-EDTA TE buffer or D1 to the dried beads. Mix thoroughly to ensure all the 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 15 µL of the eluate, ensuring as few beads as possible are carried over; transfer to a fresh set of PCR tubes and place on ice.

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

V. Protocol

G. 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 7. Final Repair Master Mix

REAGENT	FINAL REPAIR BUFFER MIX (PURPLE: FR1 VER 4)	FINAL REPAIR ENZYME MIX (PURPLE: FR2)
STORAGE	–20 °C	–20 °C
1X REACTION VOLUME	4.5 µL	0.5 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 and FR2 in a 0.5 mL capped tube, according to the volumes shown in Table 7.
5. Add 5 µL of the Final Repair Master Mix to each sample tube for a total of 20 µL.
6. Mix by pipetting, cap and spin tubes and place on ice.
7. Place the tubes in a pre-warmed thermal cycler programmed to run Program 3 (Final Repair; see Table 3):

   60 °C – 10 min, hold at 4 °C
8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

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

9. If using the True Methyl oxBS Module, continue with section H. DNA Purification and Denaturation. For alternative bisulfite conversion methods, perform bisulfite conversion according to the manufacturer recommendations and proceed to protocol section **L. Library Amplification Optimization with qPCR**.

Important: Bisulfite conversion is critical for successful library amplification.

V. Protocol

H. 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 8. 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 8.

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 30 µL water 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 NGS library 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.
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.

V. Protocol

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.

I. 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 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 B.

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.

J. 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 MOCK oxBS workflow is treated with the same aliquot of Bisulfite Reagent Solution. Mix by pipetting, spin down and place at room temperature.
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 K. Bisulfite-Converted DNA Desulfonation and Purification while the samples are in the centrifuge.

K. 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 9. 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 10.

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 oxBS-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 uL. 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 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 Optimzation 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

L. 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 10. Library Amplification qPCR Master Mix

REAGENT	AMPLIFICATION PRIMER MIX (RED: P2 VER 8)	AMPLIFICATION ENZYME MIX (RED: P3 VER 3)	20 x EvaGreen
STORAGE	–20 °C	–20 °C	4 °C
1X REACTION VOLUME	1.0 µL	4.5 µL	0.5 µL

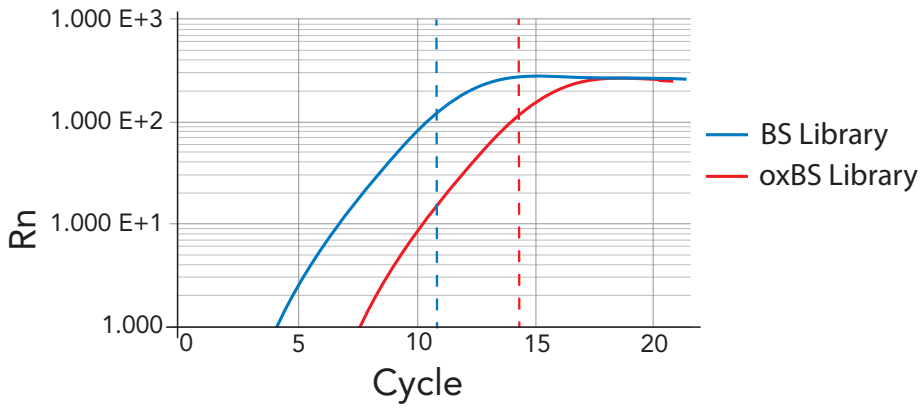
1. Prepare a master mix by combining P2, P3 and 20x EvaGreen in an appropriately sized capped tube according to the volumes shown in Table 11. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.
2. Aliquot 6 µL of master mix per sample into an 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 5**, this is 11 cycles the 'BS Library' (blue dotted vertical line, left) and 14 cycles in the 'oxBS Library' (red dotted vertical line, right).

Figure 5. Stylized qPCR amplification plot



V. Protocol

M. Library Amplification

1. Remove Agencourt beads from 4 °C. Place on the bench top to reach room temperature for use in the next step.

Table 11. 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 µL

2. Thaw P3 on ice if needed. Spin down and place on ice.
3. Thaw P2 at room temperature. Mix by vortexing, spin and place on ice.
4. Prepare a master mix by combining P2 and P3 in an appropriately sized capped tube according to the volumes shown in Table 11. Add P3 at the last moment and mix well by pipetting, taking care to avoid bubbles. Spin down and place on ice.
5. On ice, add 30 µL of Library Amplification Master Mix to each 20 µL of reserved sample for a total of 50 µL.
6. Mix well by pipetting, spin down and place on ice.
7. 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
8. Remove the tubes from the thermal cycler, spin to collect condensation and place on ice.

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

N. Amplified Library Purification

1. Ensure the Agencourt beads 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 the binding buffer and discard it.

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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 Low-EDTA TE Buffer 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.

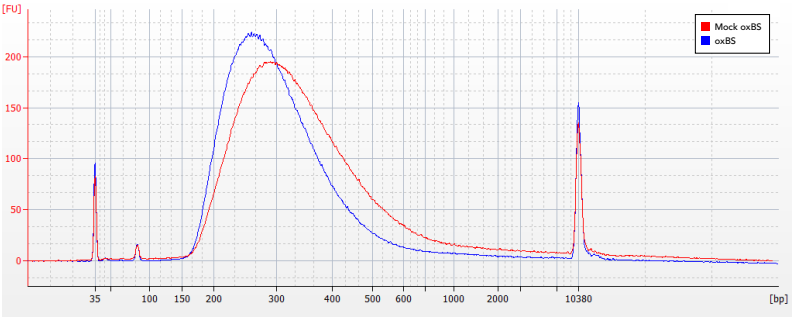
Optional stopping point: Store samples at –20 °C.

O. Quantitative and Qualitative Assessment of the Library

1. Quantify the libraries using a fluorometric assay and/or qPCR.
2. Validate libraries using the hsDNA kit for BioAnalyzer or an equivalent assay. An example trace using 1 uL of 5 ng/uL library on the hsDNA chip is shown in **Figure 6** below.

V. Protocol

Figure 6. Fragment distribution when 1 µL of 5 ng/µL library is loaded into a High Sensitivity DNA assay from 200 ng human DNA input.



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 +31.13.5780215 (Phone) 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 Multiplexing

Barcode sequences and multiplexing guidelines for adaptors used in the Ultralow Methyl-Seq library prep kit can be found in Tables Table 12 and Table 13.

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.

Table 12. Barcode sequences for adaptors used in the Ultralow Methyl-Seq 32-plex Adaptor Plate (S02694, Part No. 0366)

Barcodes in the Ultralow Methyl-Seq 32-plex Adaptor Plate are color balanced in pairs (i.e. A01 + B01, C01 + D01, etc.) and in sets of 8 by column.

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CGCTACAT	A03	GTACACCT
B01	AATCCAGC	B03	ACGAGAAC
C01	CGTCTAAC	C03	CGACCTAA
D01	AACTCGGA	D03	TACATCGG
E01	GTCGAGAA	E03	ATCGTCTC
F01	ACAACAGC	F03	CCAACACT
G01	ATGACAGG	G03	TCTAGGAG
H01	GCACACAA	H03	CTCGAACA
A02	CTCCTAGT	A04	ACGGACTT
B02	TCTTCGAC	B04	CTAAGACC
C02	GACTACGA	C04	AACCGAAC
D02	ACTCCTAC	D04	CCTTAGGT
E02	CTTCCTTC	E04	CCTATACC
F02	ACCATCCT	F04	AACGCCTT
G02	CGTCCATT	G04	TCCATTGC
H02	AACTTGCC	H04	CAAGCCAA

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Table 13. Barcode sequences for adaptors used in the Ultralow Methyl-Seq 96-Plex Adaptor Plate (S02169, Part No. 9113)

Barcodes in the Ultralow Methyl-Seq 96-plex Adaptor Plate are color balanced in sets of 8 by column.

PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE	PLATE POSITION	BARCODE SEQUENCE
A01	CACGTCTA	A05	ATGGCGAT	A09	CAAGAAGC
B01	AGCTAGTG	B05	AACGCCTT	B09	CTGATGAG
C01	ACTATCGC	C05	GTAAGGTG	C09	CTCGAACA
D01	GCGTATCA	D05	TGTCGACT	D09	TCGACAAG
E01	ACTCTCCA	E05	ACTCTGAG	E09	AGTGCATC
F01	CGTCCATT	F05	GATGGAGT	F09	TGGCTACA
G01	AGCCGTAA	G05	CTAGCTCA	G09	GCACACAA
H01	GAGTAGAG	H05	CTGTACCA	H09	GCATAGTC
A02	ACGTCGTT	A06	CCTGTCAA	A10	AACACGCT
B02	GTCCTGTT	B06	GGTCGTAT	B10	TTCACGGA
C02	AGAAGCCT	C06	CGCTGATA	C10	TGCTGTGA
D02	GAAGATCC	D06	TAGCTTCC	D10	CCTCGAAT
E02	TAGCTGAG	E06	CAAGTCGT	E10	TGTGGCTT
F02	ACGTCCAA	F06	GTCTCATC	F10	CTGTGGTA
G02	CACACATC	G06	ACCAAGCA	G10	TCACTCGA
H02	CGGATCAA	H06	AGTCAGGT	H10	ACTCCTAC
A03	TCAGCCTT	A07	TATCGCGA	A11	CCACAACA
B03	AAGGCTCT	B07	TAGCAGGA	B11	CCGCTTAA
C03	TGTTCCGT	C07	AGAAGGAC	C11	GTGGTATG
D03	GGAATGTC	D07	TGAGCTGT	D11	GGTGTACA
E03	CATCCAAG	E07	CAGAGTGA	E11	TCTAGGAG
F03	GTCAACAG	F07	AGGTTCCT	F11	TGGAAGCA
G03	TCGCTATC	G07	AGACCTTG	G11	AACACCAC
H03	AGCCTATC	H07	CTTCCTTC	H11	CATACGGA
A04	TCGGATTC	A08	CAGGTTCA	A12	CTCTCAGA
B04	CGGAGTAT	B08	ACTGGTGT	B12	AAGCTGGT
C04	GAACCTTC	C08	GGATTCAC	C12	GTGTCCTT
D04	AGAGGATG	D08	CACGATTC	D12	AGGTCTGT
E04	ACGCTTCT	E08	AGACATGC	E12	CATTCGTC
F04	CACAGGAA	F08	GACACAGT	F12	CTCACCAA
G04	ACGAATCC	G08	CCAGTTGA	G12	GACTACGA
H04	CCTTCCAT	H08	CATGGATC	H12	ATACGCAG

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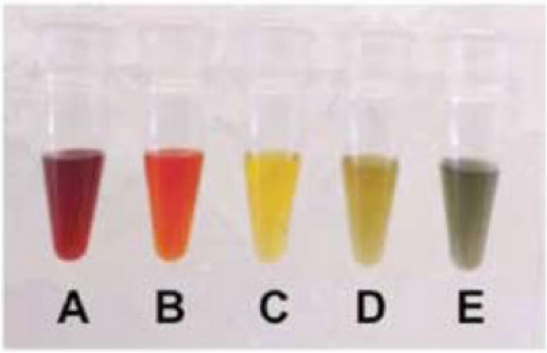
B. Oxidant Color Changes

Upon receipt of the kit, the color of the thawed Oxidant Solution should resemble Solution A in **Figure 7**. 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 7. Expected Oxidant Solution Color Changes



A. Oxidant Solution stock concentration supplied in the TrueMethyl oxBS Module.
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. I.), the color of the oxidation reaction should be similar to Solution B or C in **Figure 7**. 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 regarding oxidation solution sensitivity in section III. D. 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. J.). This is normal and will not impact downstream processing.

C. Data Analysis

Data analysis recommendations can be found using the link below.

https://github.com/nugentechnologies/NuMetWG

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D. 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 the Ultralow Methyl-Seq with TrueMethyl oxBS kit (Part Nos. 0541-32 and 9513-A01).

Input Recommendations

Q2. Can I use FFPE or other degraded DNA as input into the Ultralow Methyl-Seq library preparation kit?
Although not recommended, it is possible to generate libraries from less than 10 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.

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.

Q4. Does Tecan provide reagents for performing the fragmentation step of the protocol?
We suggest the Covaris instrument be utilized for DNA fragmentation, as suggested in the "Materials" section of this user guide. Tecan does not provide the reagents used in the fragmentation steps.

Q5. I don't have access to a Covaris instrument. Can I use alternative fragmentation methods?
We have evaluated only Covaris fragmented DNA during the development of the Ultralow Methyl-Seq library prep kit. Other mechanical means of fragmentation, such as hydrodynamic shearing or nebulization, may be suitable.

Q6. 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.

Q7. 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.

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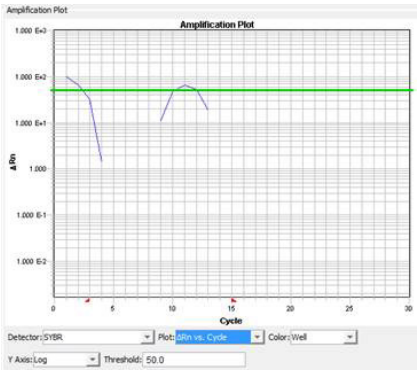
Q8. Why doesn't my Library Amplification qPCR plot resemble the example in the user guide?
Certain real-time PCR instruments may display unexpected results, such as the example in Figure 8. Ensure that your plot is set to display Rn. vs. Cycle, not deltaRn vs. Cycle, and that the y-axis is set to a log scale.

Figure 8. Results from selecting Plot > deltaRn vs. Cycle.



SPRI bead purifications

Q9. 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.

Q10. 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.
- 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.

Q11. 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.

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Sequencing Recommendations

Q12. What kind of sequencing primers can I use with your library?
The design of the Ultralow Methyl-Seq library prep kit 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.

Q13. Can the Ultralow Methyl-Seq libraries be used with paired-end sequencing?
Yes, they can be used for both single end and paired-end sequencing. Special consideration should be given to the expected insert size in the paired-end assay. The expected distances between the 5´-most and 3´-most coordinates of paired-end reads will depend on the average fragment size of the insert pool.

Q14. 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.

Q15. Are the Ultralow Methyl-Seq libraries 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.

Data Analysis

Q16. What kind of error correction is used to minimize the impact of sequencing errors in the barcodes?
Each of the barcode sequences shown in Tables 12 and 13 is 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.

Q17. 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).

Q18. 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 (http://www.broadinstitute.org/igv/). Data analysis recommendations can be found here: https://github.com/nugentechnologies/NuMetWG

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Q19. 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 Ultralow Methyl-Seq library prep kit.

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