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I. Introduction

A. Background

The Ovation® Ultralow Methyl-Seq Library Systems provide a simple, fast and scalable solution for producing directional bisulfite-converted libraries for next generation sequencing, starting with as little as 10 ng of high quality genomic DNA. The library construction workflow is designed specifically for whole genome bisulfite sequencing on Illumina NGS platforms.

The Ovation Ultralow Methyl-Seq Library Systems are available as single kits or as bundles with the TrueMethyl oxBS Module, to enable multiplexing on the Illumina sequencing platforms. The Ovation Ultralow Methyl-Seq DR Multiplex System 1–8 (Part No. 0335) and Ovation Ultralow Methyl-Seq DR Multiplex System 9–16 (Part No. 0336) each provide eight unique barcoded adaptors for multiplex sequencing. In combination, these kits enable up to 16-plex sequencing using a dedicated read (DR) barcode design. Each kit provides sufficient reagents to generate four libraries from each of the eight barcodes.

TrueMethyl® oxBS Module

The Ovation Ultralow Methyl-Seq System features the optional TrueMethyl® oxBS Module (Part No. 0414) for inputs of 10-300 ng high quality genomic DNA. The TrueMethyl oxBS module allows for the interrogation of both 5-hydroxymethylcytosine (5hmC) and 5-methylcytosine (5mC), 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)). The TrueMethyl oxBS module also includes the necessary reagents for bisulfite conversion of the libraries.
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. oxBS

The Ovation® Ultralow Methy-Seq DR Multiplex Systems 1-8 and 9-16 are fully compatible with the TrueMethyl® oxBS Module, and can be purchased as bundles (Part No. 0535 and 0536).
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, 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 cluster formation and either single read or paired-end sequencing.

Figure 2. Schematic of the Ovation Ultralow Methyl-Seq Library Systems workflow.

C. Performance Specifications

The Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16 are designed to produce DNA libraries suitable for either single read or paired-end sequencing on Illumina NGS platforms using as
I. Introduction

little as 10 ng of high quality genomic DNA as input. This system generates libraries suitable for cluster generation in less than two days.

D. Quality Control

Every lot of the Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16 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 Ultralow Methyl-Seq DR Multiplex Systems 1-8 and 9-16

The Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16 are 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

The TrueMethyl oxBS Module is shipped in two separate boxes and separately from the Ovation Ultralow Methyl-Seq DR Multiplex Systems.

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 and are stable until the expiry date provided on the TrueMethyl oxBS Module box label.

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 CO₂ 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/ngs/ovation-ultralow-methyl-seq-system.

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 Ultralow Methyl-Seq Library Systems page at NuGEN.com (www.nugen.com/products/ngs/ovation-ultralow-methyl-seq-system).

New to NGS? Contact NuGEN Technical Support at techserv@nugen.com for tips and tricks on getting started.
## Components

### A. Reagents Provided

Table 1. Ovation Ultralow Methyl-Seq DR Multiplex System 1–8 Reagents (Part No. 0335)

<table>
<thead>
<tr>
<th>PART NUMBER</th>
<th>DESCRIPTION</th>
<th>VIAL LABEL</th>
<th>VIAL NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>S01758</td>
<td>End Repair Buffer Mix</td>
<td>Blue</td>
<td>ER1 VER 8</td>
</tr>
<tr>
<td>S01533</td>
<td>End Repair Enzyme Mix</td>
<td>Blue</td>
<td>ER2 VER 4</td>
</tr>
<tr>
<td>S01709</td>
<td>End Repair Enhancer</td>
<td>Blue</td>
<td>ER3 VER 2</td>
</tr>
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<td></td>
<td>L2V11DR-BC2</td>
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<td></td>
<td>L2V11DR-BC3</td>
</tr>
<tr>
<td>S01744</td>
<td></td>
<td></td>
<td>L2V11DR-BC4</td>
</tr>
<tr>
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<td>FR1 VER 4</td>
</tr>
<tr>
<td>S01569</td>
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<td>FR2</td>
</tr>
<tr>
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<td>Red</td>
<td>P2 VER 8</td>
</tr>
<tr>
<td>S01740</td>
<td>Amplification Enzyme Mix</td>
<td>Red</td>
<td>P3 VER 3</td>
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<tr>
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<td>Orange</td>
<td>MetSeq Primer 1</td>
</tr>
<tr>
<td>S01001</td>
<td>Nuclease-free Water</td>
<td>Green</td>
<td>D1</td>
</tr>
<tr>
<td>S01502</td>
<td>Agencourt Beads</td>
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## Components

Table 2. Ovation Ultralow Methyl-Seq DR Multiplex System 9–16 Reagents (Part No. 0336)

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<tr>
<td>S01533</td>
<td>End Repair Enzyme Mix</td>
<td>Blue</td>
<td>ER2_Ver 4</td>
</tr>
<tr>
<td>S01709</td>
<td>End Repair Enhancer</td>
<td>Blue</td>
<td>ER3_Ver 2</td>
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<tr>
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<td>Ligation Buffer Mix</td>
<td>Yellow</td>
<td>L1_Ver 4</td>
</tr>
<tr>
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<td>Ligation Adaptor Mix</td>
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<td>L2V11DR-BC9</td>
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<tr>
<td>S01750</td>
<td></td>
<td></td>
<td>L2V11DR-BC10</td>
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<td>S01751</td>
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<td>S01759</td>
<td>Final Repair Buffer Mix</td>
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<td>FR1_Ver 4</td>
</tr>
<tr>
<td>S01569</td>
<td>Final Repair Enzyme Mix</td>
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<td>FR2</td>
</tr>
<tr>
<td>S01739</td>
<td>Amplification Primer Mix</td>
<td>Red</td>
<td>P2_Ver 8</td>
</tr>
<tr>
<td>S01740</td>
<td>Amplification Enzyme Mix</td>
<td>Red</td>
<td>P3_Ver 3</td>
</tr>
<tr>
<td>S01757</td>
<td>25 μM Sequencing Primer</td>
<td>Orange</td>
<td>MetSeq Primer 1</td>
</tr>
<tr>
<td>S01001</td>
<td>Nuclease-free Water</td>
<td>Green</td>
<td>D1</td>
</tr>
<tr>
<td>S01502</td>
<td>Agencourt Beads</td>
<td>Clear</td>
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</tr>
</tbody>
</table>
II. Components

Table 3. TrueMethyl oxBS Module (Part No. 0414)

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th>VIAL LABEL</th>
<th>VIAL NUMBER</th>
</tr>
</thead>
<tbody>
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<td>Oxidant Solution</td>
<td>—</td>
<td>TM-03-013</td>
</tr>
<tr>
<td>Denaturing Solution</td>
<td>White</td>
<td>TM-03-003</td>
</tr>
<tr>
<td>Ultra Pure Water</td>
<td>—</td>
<td>TM-03-002</td>
</tr>
<tr>
<td>Bisulfite Reagent Aliquot</td>
<td>—</td>
<td>TM-03-004</td>
</tr>
<tr>
<td>Bisulfite Diluent</td>
<td>—</td>
<td>TM-03-007</td>
</tr>
<tr>
<td>Desulfonation Buffer Concentrate</td>
<td>—</td>
<td>TM-03-005</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>—</td>
<td>TM-03-011</td>
</tr>
<tr>
<td>Magnetic Bead Solution</td>
<td>Clear</td>
<td>TM-03-008</td>
</tr>
<tr>
<td>Binding Buffer 1</td>
<td>Green</td>
<td>TM-03-009</td>
</tr>
<tr>
<td>Binding Buffer 2</td>
<td>Red</td>
<td>TM-03-010</td>
</tr>
</tbody>
</table>

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

- **Supplies and Labware**
  - Nuclease-free pipette tips with aerosol barriers
II. Components

- 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
III. Planning the Experiment

A. Input DNA Requirements

DNA Quantity
The Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16 are designed to work with inputs of 10–300 ng of fragmented genomic DNA. For large genomes, such as human, inputs less than 50 ng may result in lower complexity libraries. For smaller genomes, libraries can successfully be made from inputs lower than 50 ng.

<table>
<thead>
<tr>
<th>Workflow</th>
<th>Minimum Input, Complex Genomes</th>
<th>Minimum Input, Small Genomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bisulfite only</td>
<td>50 ng</td>
<td>10 ng</td>
</tr>
<tr>
<td>Oxidation + Bisulfite</td>
<td>100 ng</td>
<td>100 ng</td>
</tr>
</tbody>
</table>

Quantitation of gDNA by a dsDNA assay, such as PicoGreen® or the Qubit System, is highly recommended. Do not rely solely on OD260 readings for quantitation of input material.

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 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 NuGEN technical support (techserv@nugen.com) for specific recommendations for using challenging samples and low inputs.
III. Planning the Experiment

B. Preparation of Desulfonation Buffer for the TrueMethyl oxBS Module

1. The first time using a new kit, prepare the desulfonation buffer by adding 37.5 mL of 100% Ethanol to 16 mL of Desulfonation Buffer Concentrate

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 correctly as described. Failure to do so may result in gross loss of sample due to elution of the DNA from the beads during the Desulfonation step.
   - Store the prepared Desulfonation Buffer at 4 °C. Long-term storage of the prepared Desulfonation Buffer in the fridge is essential to prevent ethanol evaporation.

C. 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 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.
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.

D. Amplified Library Storage

Amplified libraries may be stored at –20 °C.

E. Using Ovation Ultralow Methyl-Seq on Illumina NGS Platforms

Libraries generated with the Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9-16 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 Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9-16 require the use of a custom Read 1 sequencing primer, MetSeq Primer 1, included in this kit at a concentration of 25 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. If you require additional primer, please contact technical support.

- The Standard 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 Ovation Ultralow Methyl-Seq DR Multiplex Systems produce 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. These recommendations differ by instrument. Please refer to the Illumina technical support for your instrument for more information.
III. Planning the Experiment

F. Data Analysis and Parsing Multiplex Libraries

For the Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16, follow the recommendations in the Illumina technical support documentation on parsing barcodes. The sequences of the Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16 barcodes must be entered prior to parsing. These sequences are found in Appendix A. With bisulfite-converted libraries, we notice a slightly higher rate of unmatched barcodes 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.
A. Overview

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

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. End repair</td>
<td>0.75 h</td>
</tr>
<tr>
<td>2. Adaptor ligation and purification</td>
<td>1.25 h</td>
</tr>
<tr>
<td>3. Final repair</td>
<td>0.25 h</td>
</tr>
<tr>
<td>4. DNA oxidation</td>
<td>2.0 h</td>
</tr>
<tr>
<td>5. Bisulfite conversion</td>
<td>2.0 h</td>
</tr>
<tr>
<td>6. Desulfonation and purification</td>
<td>1.5 h</td>
</tr>
<tr>
<td>7. Amplification and purification</td>
<td>1.5 h</td>
</tr>
<tr>
<td><strong>Total time to prepare library</strong></td>
<td><strong>9.25 h</strong></td>
</tr>
</tbody>
</table>

Components in the Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16 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 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 NuGEN 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 Ultralow Methyl-Seq Library Systems 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

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 4 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 4. Thermal Cycler Programming

<table>
<thead>
<tr>
<th>END REPAIR</th>
<th>VOLUME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Program 1 End Repair</td>
<td>25 °C – 30 min, 70 °C – 10 min, hold at 4 °C</td>
</tr>
<tr>
<td>ADAPTOR LIGATION</td>
<td>VOLUME</td>
</tr>
<tr>
<td>Program 2 Ligation</td>
<td>25 °C – 30 min, 70 °C – 10 min, hold at 4 °C</td>
</tr>
<tr>
<td>FINAL REPAIR</td>
<td>VOLUME</td>
</tr>
<tr>
<td>Program 3 Final Repair</td>
<td>60 °C – 10 min, hold at 4 °C</td>
</tr>
<tr>
<td>OXIDATION</td>
<td>VOLUME</td>
</tr>
<tr>
<td>Program 4 Denaturation</td>
<td>37 °C – 5 min, hold at 25 °C</td>
</tr>
<tr>
<td>BISULFITE CONVERSION</td>
<td>VOLUME</td>
</tr>
<tr>
<td>Program 5 Bisulfite Conversion</td>
<td>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</td>
</tr>
<tr>
<td>AMPLIFICATION</td>
<td>VOLUME</td>
</tr>
<tr>
<td>Program 6 Library Amplification</td>
<td>95 °C – 2 min, N(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s), hold at 10 °C</td>
</tr>
</tbody>
</table>

Note: qPCR is recommended to determine the appropriate number of PCR cycles. For more information, contact NuGEN 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.

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-300 ng) will be processed through oxBS and the other aliquot (100 ng-300 ng) will be processed through a 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.

- For alternate workflows without the TrueMethyl oxBS Module, substitute the 3rd party bisulfite treatment for steps V.H. - V.K. in the protocol. Step V.M. Library Amplification is designed to accommodate a volume of 20uL of bisulfite converted, desulfonated DNA for PCR enrichment.

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 NuGEN and will require validation. For alternatives to the Covaris system, please contact NuGEN Technical Support. The protocol below, for fragmenting DNA to ~200 bp using a Covaris S-series instrument, has been successfully used at NuGEN 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 A.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.

3. After fragmentation, briefly spin the Covaris microTUBE 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.
V. Protocol

Table 5. Covaris S-Series System Settings

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

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. (An excess of beads is provided; therefore, it is not necessary to recover any trapped in the cap.)

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 130 µL of the binding buffer and discard it. Leaving some buffer behind minimizes bead loss at this step.

   **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.

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.
V. Protocol

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 6. End Repair Master Mix

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>END REPAIR BUFFER MIX (BLUE: ER1 VER 8)</th>
<th>END REPAIR ENZYME MIX (BLUE: ER2 VER 4)</th>
<th>END REPAIR ENHANCER (BLUE: ER3 VER 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STORAGE</td>
<td>−20 °C</td>
<td>−20 °C</td>
<td>−20 °C</td>
</tr>
<tr>
<td>1X REACTION VOLUME</td>
<td>2.0 μL</td>
<td>0.5 μL</td>
<td>0.5 μL</td>
</tr>
</tbody>
</table>

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 6.
5. Add 3 μL of End Repair Master Mix to each sample tube for a total of 16 μL.
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 4): 25 °C – 30 min, 70 °C – 10 min, hold at 4 °C
V. Protocol

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 7. Ligation Master Mix

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>NUCLEASE-FREE WATER (GREEN: D1)</th>
<th>LIGATION BUFFER MIX (YELLOW: L1 ver 4)</th>
<th>LIGATION ENZYME MIX (YELLOW: L3 ver 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STORAGE</td>
<td>---</td>
<td>-20 °C</td>
<td>-20 °C</td>
</tr>
<tr>
<td>1X REACTION VOLUME</td>
<td>4.5 µL</td>
<td>6 µL</td>
<td>1.5µL</td>
</tr>
</tbody>
</table>

2. Spin down L3 and place on ice.

3. Thaw adaptor mixes (yellow: L2V11DR-BC1 through L2V11DR-BC16) on ice, spin down, and return to ice.

4. Thaw L1 and L2 at room temperature. Mix by vortexing, spin down and place on ice.

5. Add 3 µL of the appropriate L2 Ligation Adaptor Mix to each sample. Mix thoroughly by pipetting.

   **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 7. 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 tube 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 4):

   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.

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.
V. Protocol

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 65 μL of the binding buffer and discard it. Leaving some of the volume behind minimizes bead loss at this step.

**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 (Green Cap) and Binding Buffer 2 (Red Cap) from 4 °C and place at room temperature for use in the next step.

Table 8. Final Repair Master Mix

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>FINAL REPAIR BUFFER MIX (PURPLE: FR1 ver 4)</th>
<th>FINAL REPAIR ENZYME MIX (PURPLE: FR2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STORAGE</td>
<td>–20 °C</td>
<td>–20 °C</td>
</tr>
<tr>
<td>1X REACTION VOLUME</td>
<td>4.5 µL</td>
<td>0.5 µL</td>
</tr>
</tbody>
</table>

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 8.
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 4):
   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 9. Magnetic Bead Binding Solution 1 Master Mix

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>BINDING BUFFER 1 (GREEN CAP)</th>
<th>MAGNETIC BEAD SOLUTION (CLEAR CAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STORAGE</td>
<td>4 °C</td>
<td>4 °C</td>
</tr>
<tr>
<td>1X REACTION VOLUME</td>
<td>120 µL</td>
<td>2.4 µL</td>
</tr>
</tbody>
</table>

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 (Clear) and Binding Buffer 1 (Green) have reached room temperature before proceeding.

5. Mix Binding Buffer 1 by inversion until homogenized.


7. Prepare a master mix of Magnetic Bead Binding Solution 1 (MBBS1) as directed in Table 9.

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 4):
   
   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 4):
    
    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 (Red Cap), Magnetic Bead Solution (Clear Cap) and Elution Buffer from storage and place at room temperature for a minimum of 30 minutes before use.

Table 10. Magnetic Bead Binding Solution 2 Master Mix

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>BINDING BUFFER 2 (RED CAP)</th>
<th>MAGNETIC BEAD SOLUTION (CLEAR CAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STORAGE</td>
<td>4 °C</td>
<td>4 °C</td>
</tr>
<tr>
<td>1X REACTION VOLUME</td>
<td>200 µL</td>
<td>2.4 µL</td>
</tr>
</tbody>
</table>

2. Prepare a fresh stock of 70% Ethanol. Mix by vortexing or inversion.


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 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 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 23 μL Elution Buffer directly onto the bead pellet. Resuspend the beads completely by pipetting.

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 22 μL eluate into a fresh 0.2 mL tube. This is the recovered TrueMethyl converted DNA sample.
V. Protocol

L. Library Amplification Optimization with qPCR

Table 11. Library Amplification qPCR Master Mix

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>AMPLIFICATION PRIMER MIX (RED: P2 ver 8)</th>
<th>AMPLIFICATION ENZYME MIX (RED: P3 ver 3)</th>
<th>20 x EvaGreen</th>
</tr>
</thead>
<tbody>
<tr>
<td>STORAGE</td>
<td>–20 °C</td>
<td>–20 °C</td>
<td>4 °C</td>
</tr>
<tr>
<td>1X REACTION VOLUME</td>
<td>1.0 µL</td>
<td>4.75 µL</td>
<td>0.5 µL</td>
</tr>
</tbody>
</table>

1. Aliquot 2 µL of each sample into a fresh set of 0.2 mL tubes. Reserve the remaining 20 µL of sample on ice.

2. Add 14 µL of DR1 to each sample for a total of 16 µL. Mix well by pipetting, spin down and place on the bench top.

3. Aliquot 3.75 µL of each sample into an appropriate PCR plate or optically clear strip tubes, in triplicate. Mix well by pipetting, spin down and place on ice.

4. 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.

5. On ice, add 6.25 µL of Library Amplification qPCR Master Mix to each 3.75 µL of sample, in triplicate, for a total of 10 µL per replicate.

6. Mix well by pipetting, spin down and place on ice.

7. Perform qPCR with the following cycling conditions:

   95 °C – 2 min, 35x(95 °C – 15 s, 60 °C – 1 min, 72 °C – 30 s)

8. Examine the log fluorescence vs. cycle number plot from the qPCR system to determine the appropriate number of library amplification cycles.

   a. First, select a cycle number within the late exponential phase of the amplification plot. In the example in Figure 4, this is 11 cycles the ‘BS Library’ (blue dotted vertical line, left) and 14 cycles in the ‘oxBS Library’ (red dotted vertical line, right).

   b. Next, subtract 3 from the selected cycle to determine the number of PCR cycles to perform in the next step (M. Library Amplification). This compensates for the 1:8 dilution of sample used in the qPCR reaction. In the example in Figure 5 below, this is 11-3 = 8 cycles of PCR for the ‘BS Library’, and 14-3=11 cycles of PCR for the ‘oxBS Library’.
V. Protocol

M. Library Amplification

1. Remove Agencourt beads from 4 °C and DR1 from -20 °C. Place on the bench top to reach room temperature for use in the next step.

Table 12. Library Amplification Master Mix

<table>
<thead>
<tr>
<th>REAGENT</th>
<th>AMPLIFICATION PRIMER MIX (RED: P2 ver 8)</th>
<th>AMPLIFICATION ENZYME MIX (RED: P3 ver 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>STORAGE</td>
<td>–20 °C</td>
<td>–20 °C</td>
</tr>
<tr>
<td>1X REACTION VOLUME</td>
<td>5.0 µL</td>
<td>25 µL</td>
</tr>
</tbody>
</table>

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 12. 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 4):
   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 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.

   **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.
V. Protocol

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.

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 NuGEN libraries.
VI. Technical Support

For help with any of our products, please contact NuGEN 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@nugen.com.

In Europe contact NuGEN at +31(0)135780215 (Phone) or +31(0)135780216 (Fax) or email europe@nugen.com.

In all other locations, contact your NuGEN distributor for technical support.
A. Barcode Sequences and Guidelines for Multiplexing

Barcode sequences and multiplexing guidelines for adaptors used in Ovation Ultralow Methyl-Seq DR Multiplex Systems 1–8 and 9–16 can be found in Table 13 and Table 14, respectively. These 6-nucleotide barcode adaptor 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. The barcodes in this system were carefully chosen for their ability to parse properly and for color balancing and 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 13 and Table 14), 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 Ultralow 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 Ultralow 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.

Table 13. Barcode sequences for dedicated read (DR) adaptors used in Ovation Ultralow Methyl-Seq DR Multiplex System 1–8 (Part No. 0335)

<table>
<thead>
<tr>
<th>LIGATION ADAPTOR MIX</th>
<th>BARCODE SEQUENCE</th>
<th>BARCODE PAIRING (2-PLEX)</th>
<th>BARCODE PAIRING (&gt;2-PLEX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2V11DR-BC1</td>
<td>AACCAG</td>
<td>Duplex Set 1</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC2</td>
<td>TGGTGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC3</td>
<td>AGTGAG</td>
<td>Duplex Set 2</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC4</td>
<td>GCACTA</td>
<td>Duplex Set 3</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC5</td>
<td>ACCCTCA</td>
<td>Duplex Set 4</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC6</td>
<td>GTGCTT</td>
<td>One of the duplex sets from the column to the left must be used in combination with any of the other remaining individual barcodes.</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC7</td>
<td>AAGCCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC8</td>
<td>GTCGTA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## VII. Appendix

Table 14. Barcode sequences for dedicated read (DR) adaptors used in Ovation Ultralow Methyl-Seq DR Multiplex System 9-16 (Part No. 0336)

<table>
<thead>
<tr>
<th>LIGATION ADAPTOR MIX</th>
<th>BARCODE SEQUENCE</th>
<th>BARCODE PAIRING (2-PLEX)</th>
<th>BARCODE PAIRING (&gt;2-PLEX)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L2V11DR-BC9</td>
<td>AAGAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC10</td>
<td>GGAGAA*</td>
<td>Duplex Set 5</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC11</td>
<td>AGCATG</td>
<td>Duplex Set 6</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC12</td>
<td>GAGTCA</td>
<td>Duplex Set 7</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC13</td>
<td>CGTAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC14</td>
<td>TCAGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC15</td>
<td>CACAGT</td>
<td>Duplex Set 8</td>
<td></td>
</tr>
<tr>
<td>L2V11DR-BC16</td>
<td>TTGGCA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* 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. 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 CO₂. If you suspect that this has occurred, please do not use the Oxidant Solution and contact NuGEN technical support for advice.

**Figure 7. Expected Oxidant Solution Color Changes**

A. Oxidant Solution stock concentration supplied by CEGX®.
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.
VIII. Appendix

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. 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. 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

D. Frequently Asked Questions (FAQs)

Getting Started

Q1. Does NuGEN provide reagents for performing the bisulfite conversion step of the protocol?
   The bisulfite conversion reagents are included with the purchase of product part numbers 0535-32 and/or 0536-32. These bundles include the Ovation Ultralow Methyl-Seq core kit (P/N 0335,0336) and the TrueMethyl oxBS module (P/N 0414).

Input Recommendations

Q2. Can I use FFPE or other degraded DNA as input into Ovation Ultralow Methyl-Seq Library Systems?
   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 NuGEN Technical Support (techserv@nugen.com) for specific recommendations for using challenging samples and low inputs.

General Workflow

Q3. Does NuGEN 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. NuGEN does not provide the reagents used in the fragmentation steps.
Q4. 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 Ovation Ultralow Methyl-Seq Library Systems. Other mechanical means of fragmentation, such as hydrodynamic shearing or nebulization, may be suitable.

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 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

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.
- Test the magnet with a mock purification to ensure the magnet will effectively clear the solution under the conditions in the NuGEN 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. **What is the expected yield of the amplified DNA library using the Ovation Ultralow Methyl-Seq Library Systems?**

The expected yield is at least 500 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.

**Sequencing Recommendations**

Q12. **What kind of sequencing primers can I use with your library?**

The design of the Ovation Ultralow Methyl-Seq Library Systems requires the use of a custom Read 1 sequencing primer, MetSeq Primer 1, which is included in this kit at a concentration of 25 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 Ovation Ultralow Methyl-Seq Library Systems 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.
VII. Appendix

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 Ovation Ultralow Methyl-Seq Library Systems 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 DR barcode sequences shown in Tables 13 and 14 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.

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, NuGEN 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

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 Ovation Ultralow Methyl-Seq Library Systems.
VII. Appendix

E. Update History

This document, the Ovation Methyl-Seq Library Systems User Guide (M01320 v8.1), has been updated from the previous version to address the following topics:

<table>
<thead>
<tr>
<th>Description</th>
<th>Section</th>
<th>Page(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reorganize and improve clarity the TrueMethyl oxBs Module content</td>
<td>Throughout</td>
<td>Throughout</td>
</tr>
<tr>
<td>Add FAQs to provide further clarity</td>
<td>VII</td>
<td>34</td>
</tr>
<tr>
<td>Reorganize and rewrite content for better clarity</td>
<td>Throughout</td>
<td>Throughout</td>
</tr>
</tbody>
</table>