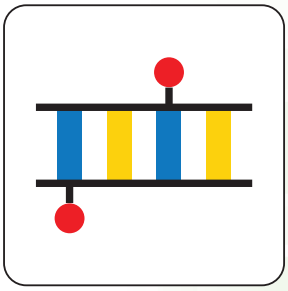
USER GUIDE



TrueMethyl® oxBS Module

Catalog Number: 0414

Publication Number: M01481

Revision: v3

NuGEN®
A Tecan Group company

For Research Use Only

Patents, Licensing and Trademarks

The purchase of this product conveys to the buyer the limited, non-exclusive, non-transferable right (without the right to modify, reverse engineer, resell, repackage or further sublicense) under these patent applications and any patents issuing from these patent applications to use this product and methods, accompanying this user guide, for research and development purposes solely in accordance with the intended use described and the written instructions provided in this user guide. No license to make or sell products by use of this product is granted to the buyer whether expressly, by implication, by estoppels or otherwise. In particular, the purchase of this product does not include or carry any right or license to use, develop or otherwise exploit this product commercially and no rights are conveyed to the buyer to use the product or components of the product for purposes including commercial services or clinical diagnostics.

For information on purchasing a license to the Tecan Genomics' patents for uses other than in conjunction with this product or to use this product for purposes other than research, please contact Tecan Genomics, Inc., 900 Chesapeake Drive, Redwood City, CA 94063. Phone 888-654-6544 or 650-590-3600; FAX 888-296-6544 or 650-590-3630.

Warranty

Tecan Genomics warrants that this product meets the performance standards described in the Company's product and technical literature for a period of six months from the date of purchase, provided that the product is handled and stored according to published instructions, and that the product is not altered or misused. If the product fails to meet these performance standards, Tecan Genomics will replace the product free of charge or issue a credit for the purchase price. Tecan Genomics' liability under this warranty shall not exceed the purchase price of the product. Tecan Genomics shall assume no liability for direct, indirect, consequential or incidental damages arising from the use, results of use or inability to use its products. Tecan Genomics reserves the right to change, alter or modify any product to enhance its performance and design.

Tecan Genomics' products are developed, designed and sold FOR RESEARCH USE ONLY. This product is not to be used for diagnostic or therapeutic purposes, nor is it to be administered to humans or animals.

Except as expressly set forth herein, no right to modify, reverse engineer, distribute, offer to sell or sell Tecan Genomics' product is conveyed or implied by buyer's purchase of this Tecan product. The buyer agrees to use Tecan products accompanying the product insert in accordance with the intended use and the written instructions provided.

Table of Contents

Contents

NuGEN
A Tecan Group company

I. Introduction

A. Background

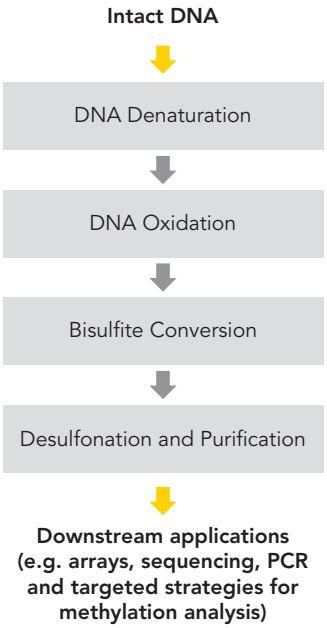
TrueMethyl® oxBS Module

The TrueMethyl oxBS Module (Part No. 0414) accommodates inputs of 100 ng – 1 µg of 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 of 5hmC bases to uracil in a complex genomic sample. The TrueMethyl oxBS Module can be used to quantitate 5hmC and can process 16 samples in parallel for oxBS and bisulfite-only to determine the 5hmC content through subtractive analysis methods. There are also sufficient reagents to process 32 individual samples through the oxBS workflow alone.

B. Workflow

As shown in Figure 1, the streamlined workflow consists of 4 main steps: DNA Denaturation, DNA Oxidation, Bisulfite Conversion, and Desulfonation and Purification of bisulfite-converted DNA.

Figure 1. TrueMethyl oxBS Workflow



With the TrueMethyl oxBS Module, parallel workflows with and without oxidation can be performed for analysis of 5hmC. The entire workflow can be completed in one day.

C. Perfomance Specifications

The TrueMethyl oxBS Module is designed to produce DNA suitable for downstream applications using 100 ng – 1 µg of high quality genomic DNA per sample as input.

I. Introduction

D. Quality Control

Every lot of the TrueMethyl oxBS Module undergoes functional testing to meet specifications for 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.

TrueMethyl oxBS Module

The TrueMethyl oxBS Module is shipped in two separate boxes.

Box 1 is shipped at room temperature.

- The Magnetic Bead Solution, Binding Buffer 1 and Binding Buffer 2 must 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.

F. Safety Data Sheet (SDS)

If appropriate, an SDS for this product is available on the NuGEN website at www.nugen.com/products/truemethyl-solutions-epigenetics.

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 TrueMethyl oxBS Module page at NuGEN.com (www.nugen.com/products/truemethyl-solutions-epigenetics).

Contact Tecan NGS Technical Support at techserv-gn@tecan.com for tips and tricks on getting started.

II. Components

A. Reagents Provided

Table 1. 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**
  - Qubit® 2.0 or 3.0 Fluorometer (Thermo Fisher Scientific) or appropriate fluorometer and accessories for quantification of fragmented DNA
  - 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)
- **Reagents**
  - HPLC grade 100% Acetonitrile (Thermo Fisher Scientific, Cat. #A998-1)
  - Ethanol (Sigma-Aldrich, Cat. #E7023), for purification steps
  - Nuclease-free water (Alfa Aesar, Cat. #J71786), for purification steps
  - Low-EDTA TE Buffer, 1X, pH 8.0 (Alfa Aesar, Cat. #J75793), for diluting nucleic acids
- **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)

II. Components

- 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)

***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

For workflows using the TrueMethyl oxBS Module, a minimum input of 100 ng per reaction is recommended.

Note: Some workflows, such as Illumina EPIC Array applications, will require a higher input of DNA. Please see section V.A. for specific recommendations on EPIC Array workflows. For other applications, contact techserv@nugen.com.

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 A260:230 ratio should be ~2.0. Using DNA samples with lower ratios may compromise your results.

DNA Integrity

Genomic DNA should be of high quality. Poor quality starting material may adversely affect results.

B. Preparation of Desulfonation Buffer for the TrueMethyl oxBS Module

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

Important:

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

III. Planning the Experiment

C. Oxidant Solution Sensitivity

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

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

Environmental sensitivities:

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

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

D. Storage

Converted (or oxidated and converted) DNA may be stored at –20 °C.

IV. Overview

A. Overview

The preparation process used in the TrueMethyl oxBS Module is performed in the following stages:

1.	DNA Oxidation	0.75 hours
2.	Bisulfite Conversion	1.25 hours
3.	Desulfonation and purification	0.25 hours
	Total time to prepare library	**2.25 hours**

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.

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 reactions 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.
- 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 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 and acetonitrile stocks to make ethanol and acetonitrile, respectively, for washes in the purification protocols.

IV. Overview

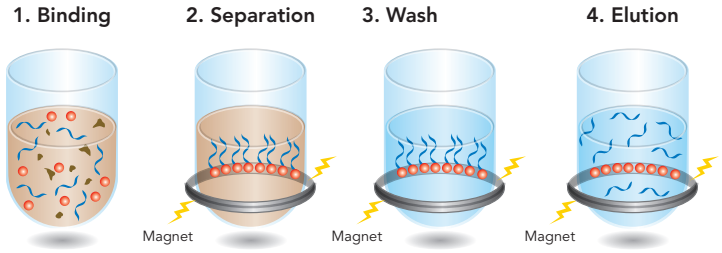
- Make the ethanol mixes fresh, carefully measuring both the ethanol and water with pipettes. Lower concentrations of ethanol in wash solutions will result in loss of yield as the higher aqueous content will dissolve the DNA and wash it off the beads or column.

C. Magnetic Beads

Magnetic beads are provided for use with the TrueMethyl oxBS Module. You must follow the protocols outlined in this user guide for the use of these beads. The bead purification process used for DNA purification before amplification consists of:

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

Figure 2. 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.

IV. Overview

- 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.

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 2 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 2. Thermal Cycler Programming

OXIDATION		**VOLUME**
Program 1 Denaturation	37 °C – 5 min, hold at 25 °C	10 µL
BISULFITE CONVERSION		**VOLUME**
Program 2 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 up to 16 hrs	40 µL

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

- For each genomic DNA sample to be analyzed for 5hmC, two independent reactions should be prepared. For each sample, one aliquot (100 ng – 1 µg) will be processed through oxBS and the other aliquot (100 ng – 1 µg) will be processed through a parallel MOCK oxBS workflow. The MOCK oxBS workflow excludes the oxidant solution.
- For bisulfite conversion without oxidation, follow the MOCK oxBS workflow.

Note: For genomic DNA to be used for EPIC Arrays, 500 ng – 1 µg should be processed through both the oxBS (if desired) and MOCK oxBS workflows.

1. Aliquot each input sample (100 ng – 1 µg) into a 0.2 mL or 0.5 mL tube.
2. Adjust each sample to 50 µL with 1X TE buffer (low EDTA) or nuclease-free water.

B. DNA Purification and Denaturation

1. Remove 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 3. 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.

V. Protocol

7. Prepare a master mix of Magnetic Bead Binding Solution 1 (MBBS1) as directed in Table 3.

Note: MBBS1 should be prepared fresh on the day of use.

8. Vortex MBBS1 master mix thoroughly to ensure the beads are homogenized in solution.
9. Add 100 µL of MBBS1 master mix to each 0.2 mL tube containing 50 µL DNA for a total of 150 µL. Mix by pipetting and centrifuge briefly.
10. Incubate at room temperature for 20 min.
11. Transfer tubes to a magnetic separation plate and incubate at room temperature for 5 minutes to completely clear the solution of beads.
12. Keeping the tubes on the magnet, carefully remove the supernatant and discard it.
13. With the tubes still on the magnet, carefully add 200 µL of 80% Acetonitrile wash to the tubes without disturbing the bead pellet.
14. Remove and discard the 200 µL 80% Acetonitrile wash, carefully avoiding aspiration of the bead pellet.
15. 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.
16. 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.

17. Remove the tubes from the magnet.
18. Add 10 µL of Denaturing Solution directly onto the bead pellet. Mix thoroughly to ensure all beads are resuspended.
19. Centrifuge briefly to collect solution at bottom of the tubes.
20. Place the tubes in a pre-warmed thermal cycler programmed to run Program 1 (Denaturation; see Table 2):

    37 °C – 5 min, hold at 25 °C
21. Remove the tubes from the thermal cycler, spin to collect condensation and transfer to the magnet.
22. Incubate at room temperature for 2 minutes.
23. 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.

V. Protocol

C. 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 C.

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 may inhibit downstream steps.
- Do not place the oxidized samples on ice as this may cause the solution to precipitate.

D. 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.
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.

V. Protocol

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.
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 2 (Bisulfite Conversion, see Table 2):

    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.

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 E. Bisulfite-Converted DNA Desulfonation and Purification while the samples are in the centrifuge.

E. 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 4. 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 4.

V. Protocol

Note:
- MBBS2 should be prepared fresh on the day of use.
- 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 15 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.
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: Ensure 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.

V. Protocol

28. Remove the tubes from the magnet.
29. Add 12.5 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 10 µL eluate into a fresh 0.2 mL tube. This is the recovered TrueMethyl converted DNA sample.

VI. Technical Support

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

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

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

VII. Appendix

A. Compatibility of TrueMethyl-Converted DNA with Downstream Workflows

TrueMethyl-converted DNA is compatible with downstream workflows including arrays, sequencing, PCR and targeted strategies for methylation. Ensure that you use the required mass of TrueMethyl-converted material in your downstream analysis platform of choice for optimal results.

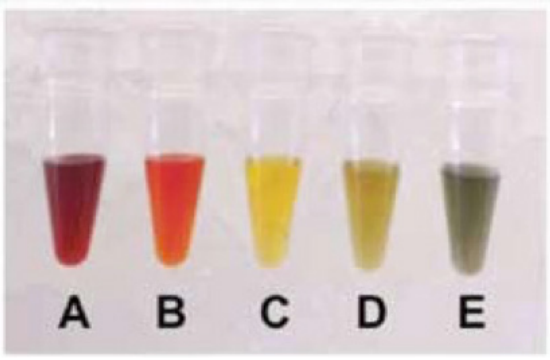
Important: Please ensure that you use the required mass of TrueMethyl converted material in your downstream analysis platform of choice for optimal results. Contact techserv@nugen.com for additional information.

For samples to be run on the Illumina Infinium® MethylationEPIC BeadChip, use 7 µL of recovered TrueMethyl template with 1 µL of 0.4 N NaOH (see Infinium methylation assay section entitled "Make MSA4 plate"). Complete all subsequent steps following the Infinium HD Assay Methylation protocol.

B. Oxidant Color Changes

Upon receipt of the kit, the color of the thawed Oxidant Solution should resemble Solution A in Figure 3. 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 NuGEN technical support for advice.

Figure 3. 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 (Step V. 3.), the color of the oxidation reaction should be similar to Solution B or C in Figure 3. 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 con-

VII. Appendix

taminants from the starting DNA sample solution. To avoid contaminants, ensure all guidance regarding oxidation solution sensitivity in section III. B. 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 (Step V. D.). This is normal and will not impact downstream processing.

C. Frequently Asked Questions (FAQs)

Input Recommendations

Q1. Can I use FFPE or other degraded DNA as input into TrueMethyl oxBS Module?
Yes. When working with FFPE or degraded DNA, we recommend inputs of 500 ng-1 µg.

General Workflow

Q2. 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.

Q3. 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.

Magnetic bead purifications

Q4. What magnetic separation devices do you recommend for the 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.

VII. Appendix

Q5. How can I ensure maximum recovery of sample from the bead purification?

- Allow the 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 or acetonitrile 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.

D. Update History

This document, the TrueMethyl oxBS User Guide (M01481 v3), has been updated from the previous version to address the following topics:

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

NuGEN®
A Tecan Group company

Tecan Genomics, Inc.

Headquarters USA
900 Chesapeake Drive
Redwood City, CA 94063 USA
Toll Free Tel: 888.654.6544
Toll Free Fax: 888.296.6544
custserv-gn@tecan.com
techserv-gn@tecan.com

Europe
P.O. Box 109,
9350 AC Leek
The Netherlands
Tel: +31-13-5780215
Fax: +31-13-5780216
europe-gn@tecan.com

Worldwide
For our international distributors contact information, visit our website
www.nugen.com