Product Manual

Blood and Tissue DNA Methylation Kit

ENZ-45004-0050

ENZ-45004-0200

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Enzo Life Sciences, Inc. is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Enzo Life Sciences, Inc., within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NON- INFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending. Enzo is a trademark of Enzo Life Sciences, Inc.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

TABLE OF CONTENTS

INTRODUCTION

DNA methylation is a naturally occurring event in both prokaryotic and eukaryotic organisms. In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression[1]. It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis[2]. DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation. In many plants and animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme[3]. The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do exist. In fact, about 80 percent of all 5'-CpG-3' dinucleotides in mammalian genomes are found to be methylated, whereas the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to detect/quantify DNA methylation including: high-performance capillary electrophoresis[4] and methylation-sensitive arbitrarily primed PCR[5]. However, the most common technique used today remains the bisulfite conversion method[6]. This technique involves treating methylated DNA with bisulfite, which converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing (see below).

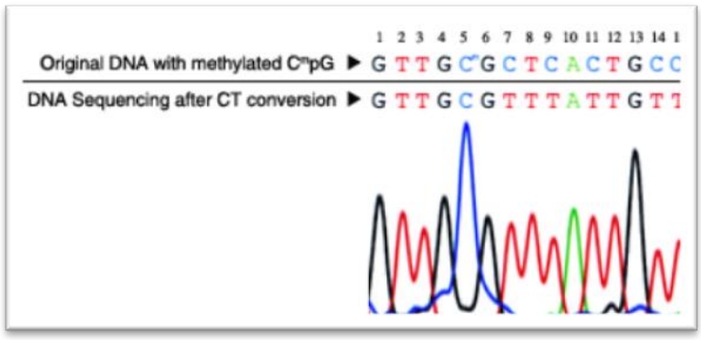

Figure 1: DNA sequencing results following bisulfite treatment. DNA with methylated $C^{m}pG$ at nucleotide position #5 was processed using the **Express DNA Methylation Kit**. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remained intact while the unmethylated cytosines at positions #7, 9, 11, 14 and 15 were completely converted into uracil following bisulfite treatment and detected as thymine following PCR.

ASSAY PRINCIPLE

The **Blood and Tissue DNA Methylation Kit** features simple and reliable DNA bisulfite conversion directly from blood, tissue, and cells without the prerequisite for DNA purification. The increased sensitivity of this kit makes it possible to amplify bisulfite converted DNA from as few as 10 cells or 50 pg DNA. The DNA denaturation and bisulfite conversion processes are combined into a single step (see below). All kits streamline the three step process of bisulfite conversion of non-methylated cytosine in DNA into uracil. In addition the methylation kits share innovative in-column desulphonation technology that eliminates otherwise cumbersome DNA precipitation steps while ensuring researchers consistent results every time. The kits have been designed to minimize template degradation, loss of DNA during treatment and clean-up, and to provide complete conversion of unmethylated cytosines. Recovered DNA is ideal for PCR amplification for downstream analyses including restriction endonuclease digestion, sequencing, microarrays, etc.

- **Conversion Efficiency:** > 99.5% of non-methylated C residues are converted to U; > 99.5% protection of methylated cytosines.
- **DNA Recovery:** > 80%
- **Sensitivity of Detection (Lower Limit):** 10 cells for successful PCR amplification.

MATERIALS SUPPLIED

Reagent	ENZ-45004-K050	ENZ-45004-K200	Storage Conditions	Format
Proteinase K and Storage Buffer*	5 mg set	20 mg set	-20ºC after mixing	Solid/Liquid
Methylation Digestion Buffer (2X)	4 mL	15 mL	RT	Liquid
BT Conversion Reagent*	5 tubes	20 tubes	RT	N/A
Methylation Dilution Buffer	1.5 mL	7 mL	RT	Liquid
Methylation-Solubilization Buffer	4.5 mL	18mL	RT	N/A
Methylation-Reaction Buffer	1 mL	4mL	RT	N/A
Methylation-Binding Buffer	30 mL	125 mL	RT	Liquid
Methylation-Wash Buffer Concentrate*	6 mL	24 mL	RT	Liquid
M-Desulphonation Buffer	10 mL	40 mL	RT	Liquid
Methylation-Elution Buffer	1 mL	4 mL	RT	Liquid
IC Columns	50 units	200 units	RT	N/A
CollectionTubes	50 tubes	200 tubes	RT	N/A

*Some reagents require preparation prior to using. Please review the reagent preparation section of the manual

STORAGE

Store reagents at room temperature conditions, unless otherwise noted.

STABILITY

12 months upon receipt

OTHER MATERIALS NEEDED

1. 100% (Absolute) Ethanol
2. PCR tube
3. PCR Instrumentation
4. Calibrated pipette
5. Microtube centrifuge (Counter)
6. -20°C or -70°C freezer
7. Designed primers (26-32 bp)
8. DNA per bisulfate reaction (200 ng- 500 ng) or from Cell sample
9. UV spectrometer
10. Reagents for PCR reaction (e.g. polymerase)

PRECAUTIONS

1. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. In case of accidental skin exposure, flush with water immediately.
2. Interpretation of the results is the sole responsibility of the user.

REAGENT PREPARATION

NOTE: *Reagents should be briefly centrifuged to ensure contents are fully collected.*

Methylation Wash Buffer

Add 24 mL of 100% ethanol to the 6 mL **Methylation Wash Buffer Concentrate** (ENZ-45004-0050) or 96 mL of 100% ethanol to the 24 mL **Methylation Wash Buffer Concentrate** (ENZ-45001-K200) before use.

Proteinase K Solution

Add 260 µL (1040 µL for ENZ-45004-0200) of **Proteinase K Storage Buffer** to the **Proteinase K**. Mix gently to produce homogenous solution. Final concentration is 20 mg/mL. After the material has dissolved completely, store at -20ºC.

BT Conversion Reagent Solution

Add 790 µL **Methytation- Solubilization Buffer** and 300 µL **Methylation- Dilution Buffer** per tube of **BT Conversion Reagent**. Mix well by frequently vortexing or shaking for 10 minutes at room temperature. Prior to use add 160 µL of **Methylation-Reaction Buffer.** Mix well.

Note: It is normal to see trace amounts of undissolved reagent in the **BT Conversion Reagent**. Each tube of **BT Conversion Reagent** is designed for 10 separate DNA treatments.

Storage: The **BT Conversion Reagent** is light sensitive, so minimize its exposure to light. For best results, the **BT Conversion Reagent** should be used immediately following preparation. If not used immediately, the **BT Conversion Reagent Solution** can be stored overnight at room temperature, one week at 4°C, or up to one month at -20°C. Stored **BT Conversion Reagent Solution** must be warmed to 37°C, then vortexed prior to use.

SAMPLE PREPARATION

Cells: Compatible with cells from solid tissue, tissue culture, whole blood, buffy coat, biopsies, LCM (Laser-Capture Micro-Dissection) and FFPE samples, etc. The number of cells per standard treatment can range from 10-10^5 cells. For optimal results, the cell number should be from 1 x 10^3-8 x 10^4 cells.

Purified DNA: Samples containing 50 pg– 2 µg of DNA. For optimal results, the amount of input DNA should be from 200 to 500 ng.

Use appropriate kit for DNA preparation. Optimal DNA amount 200 ng- 500 ng per reaction.

METHODS

The procedure described in this manual assumes that the user is familiar with the basic principles of PCR and with using proper sterile technique.

Overall Procedure

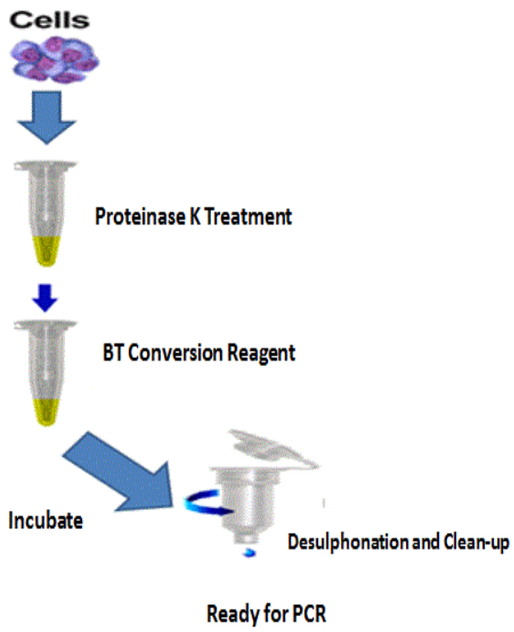

Figure 2 Outline of the **Blood and Tissue Methylation Kit** procedures. This includes treating the cells with Proteinase K followed by BT conversion incubation step.

The application protocols included might require optimization

Important: *PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING. Prepare and use appropriate desired controls for each experiment. Prior to beginning experiment, turn on the PCR equipment and set to desired parameters.*

Note: Either blood, tissue, cells, or purified DNA can be used as the starting material for the **Blood and Tissue Methylation Kit.** If blood, tissue or cells are use, see Appendix Section. For optimal results, the cell number should be between 1 x 10^3-8 x 10^4 per treatment, although the cell number can range from 10-10^5 cells. *Using more cells than the recommended limit may result in incomplete bisulfite conversion of the DNA.*

PROTOCOL

Sample Digestion with Proteinase K Solution

Digestions should be performed in a tube (e.g., PCR tube) using either procedure **1A** or **1B** (below) based on the number of cells and/or tissue type. Digestions are scalable to facilitate multiple samples or to increase the ease of manipulation. Sufficient volumes of reagents are included with this kit to increase the overall **Proteinase K** digestion volume 5-fold.

Important! "Difficult to digest" samples result in the formation of visible debris following digestion. These type of samples should be processed according to procedure **1B**.

1. Digestion Procedure (A or B)
   - **A.** Setup the following digestion for samples containing up to 2 x 10^3 cells.

10 µL	**Methylation-Digestion Buffer (2X)**
Up to 9 µL	Sample (≤ 2 x 10^3 cells)
1 µL	**Proteinase K Solution**
X µL	H_2O
20 µL	Total Volume

   - **B.** Setup the following digestion for samples containing up to 1 x 10^5 cells. This should also include all "difficult to digest" samples that form debris or precipitate following **Proteinase K** digestion– see **Appendix A**.

13 µL	Methylation Digestion Buffer (2X)
Up to 12 µL	Sample (≤ 10^5 cells)
1 µL	Proteinase K Solution
X µL	H_2O
26 µL	Total Volume

2. Incubate the sample(s) for 20 minutes at 50°C.

   Note: For FFPE, LCM and other "fixed" tissue samples, adjust the incubation time to 4 hours **(see Appendix A).**

3. If procedure **1A** was used, proceed directly to the Bisulfite Conversion of DNA procedure listed below. If following procedure **1B**, mix the contents of the reaction thoroughly then centrifuge for 5 minutes at 10,000 x *g.* Use 20 µL of the supernatant when proceeding to the bisulfite conversion section listed below.

   Note: Proteinase K digested material can be stored for several months at -20ºC.

Bisulfite Conversion of DNA

1. Add 20 µL of sample from Step 3 (**previous section**) to 130 µL of **BT Conversion Reagent Solution** in a PCR tube. Mix the sample and then centrifuge briefly to ensure no droplets are in the cap or sides of the tube.

   Note 1: Optional- If procedure **1A** was used from the (Sample Digestion procedure), the **BT-Conversion Reagent Solution** can be added directly to the samples in a PCR tube.

   Note 2: If purified DNA is used, add up to 20 µL of DNA to 130 µL of BT Conversion Reagent Solution. If the volume of DNA is less than 20 µL, compensate with water.

2. Place the PCR tube(s) in a thermal cycler and perform the following steps:

   a. 98°C for 8 minutes

   b. 64°C for 3.5 hours

   c. 4°C storage up to 20 hours

   Note: The 4°C storage step is *optional*. Additional PCR setup information can be found in the Appendix B section of the manual.

3. Add 600 µL of **Methylation-Binding Buffer** into an **IC Column** and place the column into a provided **Collection Tube**.

   Note: The capacity of the **collection tube** with the column inserted is 800 µL. Empty the collection tube whenever necessary to prevent contamination of the column contents by the flow-through.

4. Load the sample (from Step 2) into the **IC Column** containing the **Methylation-Binding Buffer**. Close the cap and mix by inverting the column several times.

5. Centrifuge at full speed (≥10,000 x *g*) for 30 seconds. Discard the flow-through.

6. Add 100 µL of **Methylation-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds.

7. Add 200 µL of **M-Desulphonation Buffer** to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds.

8. Add 200 µL of **Methylation-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds. Add another 200 µL of **Methylation-Wash Buffer** and centrifuge for an additional 30 seconds.

9. Place the column into a 1.5 mL microcentrifuge tube. Add 10 µL of **Methylation-Elution Buffer** directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA.

   Note: Alternatively, water or TE (pH ≥ 6.0) can be used for elution if required for your experiments.

   The DNA is ready for immediate analysis or can be stored at or below -20°C for later use. For long term storage, store at or below -70°C. We recommend using 1-4 µL of eluted DNA for each PCR, however, up to 10 µL can be used if necessary. The elution volume can be >10 µL depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA.

APPENDIX

A. Recommendations for Specific Cells and Tissues

The following guidelines are provided as recommendations when sampling specific cell and tissue sources. *Most importantly,* the optimal amount of DNA used for bisulfite treatment (**Section B**) should be from 1 x 10^3-8 x 10^4 cells, although DNA from as few as 10 to as many as 10^5 cells may be used. *Caution: using more cells than the recommended maximum may result in incomplete bisulfite conversion of the DNA*.

Important! "Difficult to digest" samples result in the formation of visible debris following digestion and should be processed according to digestion procedure **1. B**. This can occur with samples that are large or resistant to **Proteinase K** digestion, including: connective tissue (e.g., cartilage), adipose tissue, some fixed tissue, etc. *If debris is not removed by centrifugation, it may interfere with the bisulfite conversion process resulting in incomplete conversion of the DNA.*

Whole Blood: Use up to 0.5 µL whole blood per Proteinase K digestion (procedure **1.A** or **1.B**).However, the volume of the **Proteinase K Solution** can be adjusted when processing multiple samples or for convenient sample manipulation. For example, to increase the sample volume 5-fold for digestion procedure **A**: add 2.5 µL of blood to 50 µL **Methylation-Digestion Buffer**, 42.5 µL H_20, and 5 µL of **Proteinase K Solution**.

Solid Tissue (Fresh or Frozen): Use up to 0.1mg or 0.1 µL tissue per **Proteinase K** digestion (procedure **A** or **B)**. However, the volume of the **Proteinase K** solution can be adjusted when processing multiple samples or for convenient sample manipulation. For example, to increase the sample volume 5-fold for digestion procedure **B**: add 0.5 mg or 0.5 µL of tissue to 65 µL **Methylation-Digestion Buffer**, 59.5 µL H_20, and 5 µL of **Proteinase K Solution.**

Cultured Cells and Other Cell-Containing Liquids: Both monolayer and cells in suspension may be processed either directly from the culture container or after harvesting. Small amounts of culture medium do not adversely affect the procedure but should be kept to a minimum. Ideally, cells should be suspended in PBS or Tris-buffered solutions prior to Proteinase K digestion.

Other cell-containing liquids (e.g., those derived from FACS or buffy coat) may also be used directly as sample sources. If the composition of the liquid is not “defined”, then pellet the cells by centrifugation and remove the supernatant. Cells should be re-suspended in PBS or Tris-buffered solutions. Generally, cells in body fluids can be used directly for **Proteinase K** digestion.

FFPE (Formalin-Fixed Paraffin-Embedded) and Other “Fixed” Tissues: Paraffin-embedded tissues must be deparaffinized prior to use. This can be accomplished according to conventional xylene-ethanol protocols. The Proteinase K digestion must be extended from 20 minutes to 4 hours for FFPE and any other fixed tissue samples.

LCM (Laser Capture Micro-Dissection): Tissue samples from LCM should be in PBS or Tris-buffered solutions. The Proteinase K digestion must be extended from 20 minutes to 4 hours for LCM and any other fixed tissue samples.

B. Bisulfite Conversion and PCR Optimization

1. **Bisulfite Conversion of Double Stranded DNA Templates.** The following illustrates what occurs to a DNA template during bisulfite conversion.

Template:	**A:**	5′–GACCGTTCCAGGTCCAGCAGTGCGCT–3′
	B:	3′–CTGGCAAGGTCCAGGTCGTCACGCGA–5′
Bisulfite Converted:	**A:**	5′–GATCGTTTTAGGTTTAGTAGTGCGTT–3′
	B:	3′–TTGGCAAGGTTTAGGTTGTTATGCGA–5′

Note: Methylated “C” is underlined in the examples. Following bisulfite conversion, the strands are no longer complementary.

2. **PCR Primer Design,** Generally, primers 26 to 32 bases are required for amplification of bisulfite converted DNA. In general, all Cs should be treated as Ts for primer design purposes, unless they are in a CpG context. See example below.

Bisulfite Converted:	**A:**	5′–GATCGTTTTAGGTTTAGTAGTGCGTT–3′	
Primers: Reverse:		3′–ATCATCACRCAA–5′	**R**= G/A
Forward:		5′–GATYGTTTTAGGT–3′	**Y**= C/T

Note: Only one strand (**A**) is amplified by a given primer set. Only the reverse primer binds to the converted DNA, the forward primer will bind the strand generated by the reverse primer. If the primer contains CpG dinucleotides with uncertain methylation status, then mixed bases with C and T (or G and A) can be used. Usually, there should be no more than one mixed position per primer and it should be located toward the 5’ end of the primer. It is not recommended to have mixed bases located at the 3’ end of the primer

3. **Amount of DNA Required for Bisulfite Conversion.** The minimal amount of human or mouse genomic DNA required for bisulfite treatment and subsequent PCR amplification is 100 pg. The optimal amount of DNA per bisulfite treatment is 200 to 500 ng. Although up to 2 µg of DNA can be processed, it should be noted that high input levels of DNA may result in incomplete bisulfite conversion for some GC-rich regions.

4. **PCR Conditions.** Usually, 35 to 40 cycles are required for successful PCR amplification of bisulfite converted DNA. Optimal amplicon size should be between 150-300 bp; however larger amplicons (up to 1 kb) can be generated by optimizing the PCR conditions. Annealing temperatures between 55-60°C typically work well.

   Note: DNA polymerase specifically designed for the amplification of bisulfite treated. DNA is a "hot start" polymerase.

   As most non-methylated cytosine residues are converted into uracil, the bisulfite-treated DNA usually is AT-rich and has low GC composition. Non-specific PCR amplification is relatively common with bisulfite treated DNA due to its AT-rich nature. PCR using "hot start" polymerases is strongly recommended for the amplification of bisulfite-treated DNA.

5. **Quantifying Bisulfite Treated DNA.** Following bisulfite treatment of genomic DNA, the original base-pairing no longer exists since non-methylated cytosine residues are converted into uracil. Recovered DNA is typically A, U, and T-rich and is single stranded with limited non-specific base-pairing at room temperature. The absorption coefficient at 260 nm resembles that of RNA. Use a value of 40 µg/mL for Ab_{260} = 1.0 when determining the concentration of the recovered bisulfite-treated DNA.

REFERENCES

1. Costello JF, Plass CJ. Med. Genet. 2001; 38(5): 285- 303.
2. Stirzaker C. Cancer Res. 1997; 57(11): 2229-2237.
3. Adams RL. Bioessays. 1995; 17(2): 139-145.
4. Fraga MF, *et al*. Electrophoresis. 2000; 21(14): 2990-2994.
5. Gonzalgo ML. Cancer Res. 1997; 57(4): 594-599.
6. Frommer M. Proc. Natl. Acad. Sci. USA. 1992; 89(5): 1827-1831.

GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzolifesciences.com