Product Manual

Express DNA Methylation Kit

Catalog #: ENZ-45002-0002

(Deep-Well)

Catalog#: ENZ-45003-0002

(Shallow-Well)

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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.

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INTRODUCTION TO DNA METHYLATION

Cytosine methylation in both prokaryotic and eukaryotic organisms, consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring via a methyltransferase enzyme.[1] In prokaryotes DNA methylation provides a way to protect host DNA from digestion by restriction endonucleases that are designed to eliminate foreign DNA. DNA methylation in higher eukaryotes functions in the regulation/control of gene expression.[2]

The majority of DNA methylation in mammals occurs in 5′-CpG-3′ dinucleotides. About 80 percent of all 5′-CpG-3′ dinucleotides in mammalian genomes are found to be methylated, and the majority of the twenty percent that remain unmethylated are within promoters or in the first exons of genes. 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.[3] DNA methylation has also been shown to play a central role in gene imprinting, embryonic development, X-chromosome gene silencing, and cell cycle regulation.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, and 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 techniques used today still rely on bisulfite conversion.[6]

Treating DNA with bisulfite chemically modifies non-methylated cytosines into uracil, methylated cytosines remain unchanged. Once converted, the methylation profile of the DNA can be determined using the desired downstream application. For single locus analysis, the region of interest is generally amplified following bisulfite conversion (i.e., bisulfite PCR) and then sequenced or processed for Pyrosequencing®. Recent advances in methylation detection also allow the investigation of genome-wide methylation patterns using technologies including array-based methods, reduced representation bisulfite sequencing (RRBS), and whole genome bisulfite sequencing.[7]

ASSAY PRINCIPLE

The **Express DNA Methylation Kit (shallow well or deep well)** features high-throughput (96-well) bisulfite treatment and conversion of DNA for methylation analysis. No preparation is necessary when using the **Express Conversion Reagent.** Simply add this reagent to a DNA sample, wait about an hour, and let the reaction proceed to completion. DNA denaturation and bisulfite conversion processes are combined with added heat to facilitate rapid denaturation. Desulphonation and clean-up of the converted DNA is performed using a unique 96-well spin-plate. High yield, converted DNA is ideal for PCR, array, bisulfite and next generation sequencing.

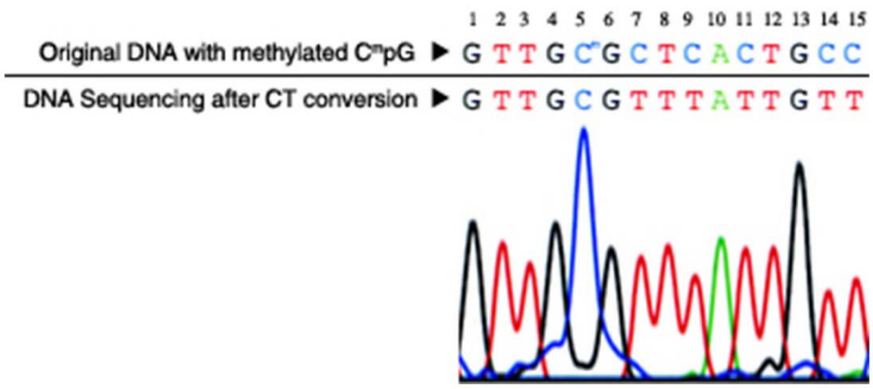

Figure 1 DNA sequencing results following bisulfite treatment. DNA with methylated C at nucleotide position #5 was processed using the **Express DNA Methylation Kit**. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remains intact while the unmethylated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

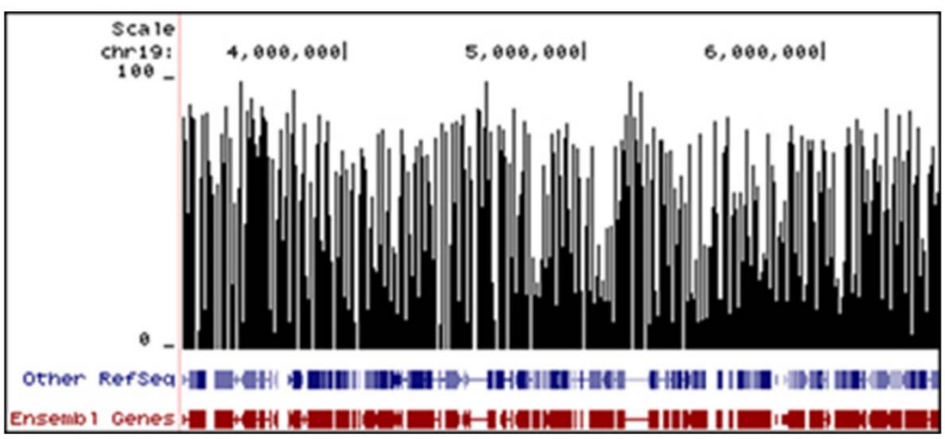

Figure 2: Data shows the relative percentage of methylation at individual CpG sites in mouse DNA (i.e. Methylation Plot from Reduced Representation Bisulfite Sequencing (RRBS). Methylation percentage is shown across a ~3 Mb region of mouse chromosome 19. Bisulfite sequencing libraries were prepared

using mouse genomic DNA prepped with a Genomic DNA cleanup column and bisulfite converted using the **Express DNA Methylation Kit** prior to Next-Gen sequencing.

CRITERIA

- **Conversion Efficiency:** > 99.5% of non-methylated C residues are converted to U; > 99.5% protection of methylated cytosines.
- **DNA Recovery:** > 80%

MATERIALS PROVIDED

Reagent	ENZ-45002	ENZ-45003	StorageTemperature
Express Conversion Reagent*	2 bottles	X	RT
Methylation Binding Buffer	125ml	X	RT
Methylation Wash Buffer Concentrate**	2 x 36ml	X	RT
L-Desulphonation Reagent	40ml	X	RT
Methylation Elution Buffer	8ml	X	RT
Express Binding Plates (Deep or Shallow)	2 Deep plates	2 Shallow Plates	RT
Express Conversion Plates	2 plates	X	RT
Collection Plates	2 plates	X	RT
Elution Plates	2 plates	X	RT

* The **Express Conversion Reagent** is in a ready-to-use liquid format. The reagent should be stored tightly capped at room temperature with minimum exposure to light.

** Add 144ml of 100% ethanol to the 36ml **Methylation Wash Buffer Concentrate** before use.

STORAGE

Store reagents at room temperature conditions.

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. -20ºC or -70ºC freezer
7. Designed primers (26-32 bp)
8. **DNA Input**-Samples containing between 100 pg to 2µg of DNA. For optimal results, the amount of input DNA should be from 200 to 500ng.
9. UV spectrometer
10. Microplate centrifuge
11. Reagents for PCR reaction (e.g. polymerase)

PRECAUTIONS

1. Wear appropriate personal protective apparel. Avoid contact with clothes and exposed skin. Wear protective gloves and eye protection. In case of accidental skin exposure, flush with water immediately.
2. This product is for research use only and should only be used by trained professionals.
3. 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 Concentrate

Add 144 mL of 100% ethanol to the 36 ml **Methylation Wash Buffer Concentrate** before use. Make sure that solution is homogenous.

Express Conversion Reagent

No preparation step is required!

DNA preparation

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

METHODS AND PROCEDURE

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

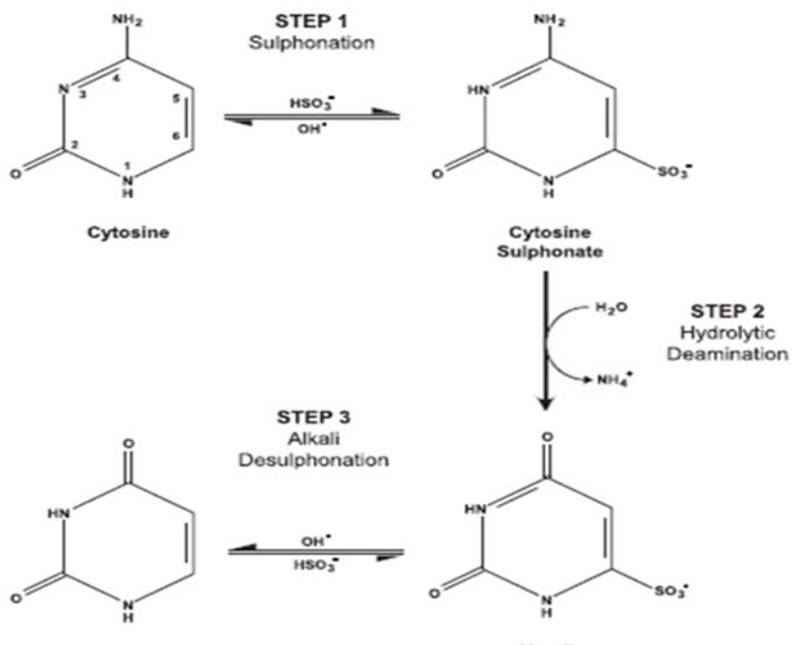

Figure 3: Overview of Bisulfite Conversion. Steps 1 and 2 occur during bisulfite conversion, while Step 3 is performed as the DNA is bound to the column matrix. For the reaction to proceed to completion, it is essential the DNA be fully denatured.

NOTE: *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.*

Important! Follow Method A if using ENZ-45002 (Deep Well) or Method B if using ENZ-45003 (Shallow Well)

METHOD A

1. Add 130µl of the **Express Conversion Reagent** to 20µl of a DNA sample in a Conversion Plate. Mix the samples by pipetting up and down.

   Note: If the volume of DNA is less than 20µl, compensate with water Samples >20µl must be processed using multiple conversion reactions. Replicate reactions can be cleaned using the same well by repeating steps 3-5.

2. Seal the plate with the provided film. Transfer the **Express Conversion Plate** to a thermal cycler and perform the following steps:

   A. 98°C for 8 minutes

   B. 54°C for 60 minutes

   C. 4°C storage for up to 20 hours

   Note: The 4°C storage step is *optional*.

3. Add 600µl of **Methylation Binding Buffer** to the wells of the **Express Binding Plate (Deep)** mounted on the **Collection Plate**.

   Note: The capacity of each well of the Binding Plate is 600µl. The capacity of each well of the Collection Plate is 800µl. Empty the Collection Plate

4. Transfer the samples from the **Express Conversion Plate** (Step 2) to the wells of the **Express Binding Plate (Deep)**. Mix by pipetting up and down.

5. Centrifuge at ≥ 3,000 x *g* (5,000 x *g* max.) for 5 minutes. Discard the flow-through.

6. Add 400µl of **Methylation Wash Buffer** to each well of the plate. Centrifuge at ≥ 3,000 x *g* for 5 minutes.

7. Add 200µl of **L-Desulphonation Reagent** to each well and allow the plate to stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at ≥ 3,000 x *g* for 5 minutes. Discard the flow-through.

8. Add 400µl of **Methylation Wash Buffer** to each well of the plate. Centrifuge at ≥ 3,000 x *g* for 5 minutes. Discard the flow-through. Add another 400µl of **Methylation Wash Buffer** and centrifuge for 10 minutes.

9. Place the **Express Binding Plate (Deep)** onto an **Elution Plate**. Add 15µl of **Methylation Elution Buffer** directly to each well. Wait 5 minutes, then centrifuge at ≥ 3,000 x *g* for 3 minutes to elute the DNA.

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 1-4µl of eluted DNA for each PCR, however, up to 30µl can be used if necessary. The elution volume can be > 15µl depending on the requirements of your experiments, but small elution volumes will yield higher DNA concentration.

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

METHOD B

1. Add 130µl of the **Express Conversion Reagent** to 20µl of a DNA sample in a Conversion Plate. Mix the samples by pipetting up and down.

2. Seal the plate with the provided film. Transfer the Express Conversion Plate to a thermal cycler and perform the following steps.

   Note: If the volume of DNA is less than 20µl, compensate with water

   Samples >20µl must be processed using multiple conversion reactions. Replicate reactions can be cleaned using the same well by repeating steps 3-5.

   A. 98°C for 8 minutes

   B. 54°C for 60 minutes

   C. 4°C storage for up to 20 hours

   Note: The 4°C storage step is *optional*.

3. Add 400µl of Methylation Binding Buffer to the wells of the Express Binding Plate (Shallow) mounted on the Express Collection Plate.

   Note: The capacity of each well of the Binding Plate is 600µl. The capacity of each well of the Collection Plate is 800µl. Empty the Collection Plate

4. Transfer the samples from the **Express Conversion Plate** (Step 2) to the wells of the **Express Binding Plate (Shallow)**. Mix by pipetting up and down.

5. Centrifuge at ≥ 3,000 x *g* (5,000 x *g* max.) for 5 minutes. Discard the flow-through.

6. Add 400µl of **Methylation Wash Buffer** to each well of the plate. Centrifuge at ≥ 3,000 x *g* for 5 minutes.

7. Add 200µl of **L-Desulphonation Reagent** to each well and allow the plate to stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at ≥ 3,000 x *g* for 5 minutes. Discard the flow-through.

8. Add 400µl of **Methylation Wash Buffer** to each well of the plate. Centrifuge at ≥ 3,000 x *g* for 5 minutes. Discard the flow-through. Add another 400µl of **Methylation Wash Buffer** and centrifuge for 10 minutes.

9. Place the **Express Binding Plate (Shallow)** onto an **Elution Plate**. Add 30µl of **Methylation Elution Buffer** directly to each well. Wait 5 minutes, then centrifuge at ≥ 3,000 x *g* for 3 minutes to elute the DNA.

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 1-4µl of eluted DNA for each PCR, however, up to 30µl can be used if necessary. The elution volume can be > 30µl depending on the requirements of your experiments, but small elution volumes will yield higher DNA concentration.

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

APPENDIX: 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.

   Note: Methylated "C" is underlined in the examples

   Template:

   A: 5′-GACCGTTCCAGGTCCAGCAGTGCGCT-3′

   B: 3′-CTGGCAAGGTCCAGGTCGTCACGCGA-5′

   Bisulfite Converted:

   A: 5′-GATCGTTTTAGGTTTAGTAGTGCGTT-3′

   B: 3′-TTGGCAAGGTTTAGGTTGTTATGCGA-5′

   Note: 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 100pg. The optimal amount of DNA per bisulfite treatment is 200 to 500ng. Although, up to 2µg 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 150-300bp; however larger amplicons (up to 1kb) can be generated by optimizing the PCR conditions. Annealing temperatures between 55-60°C typically work well.

   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.

   Note: “Hot start” DNA polymerase is required

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 260nm resembles that of RNA. Use a value of 40µg/ml for Ab_{260} = 1.0 when determining the concentration of the recovered bisulfite-treated DNA.

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