



ZYMO RESEARCH

The Beauty of Science is to Make Things Simple

INSTRUCTION MANUAL

EZ DNA Methylation-Startup™ Kit

Catalog No. **D5024**

Highlights

- Designed for the first time user requiring a consolidated product to perform DNA methylation analysis.
- A complete system for DNA methylation detection: DNA bisulfite treatment, robust hot start PCR master mix, and a universally methylated human control with primers.
- Ideal for system setup and calibration for DNA methylation detection and analysis.

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Satisfaction of all Zymo Research products is guaranteed. If you should be dissatisfied with this product please call 1-888-882-9682.

Product Contents

EZ DNA Methylation-Startup™ Kit (Format)	D5024 (1 Kit)	Storage Temperature
EZ DNA Methylation-Direct™ Kit* (Cat. #D5020)	50 Rxn. Kit	Room Temp.
Universal Methylated Human Standard (Cat. #D5011)	20 µl	-20°C
hMLH1 Primer I	20 µl	-20°C
hMLH1 Primer II	20 µl	-20°C
2X Zymo Taq™ PreMix (Cat. #E2003)	2 x 625 µl	-20°C
DNase/RNase-Free H₂O	2 x 1 ml	Room Temp.

Note - Integrity of kit components is guaranteed for one year from date of purchase. Reagents are routinely tested on a lot-to-lot basis to ensure they provide maximal performance and reliability.

*See protocol included in the kit for product contents and storage guidelines.

Specifications:

Application: Workflow design allows direct sample input for bisulfite treatment of DNA followed by robust PCR amplification of the “converted” DNA for methylation analysis.

Components: The EZ DNA Methylation-Startup™ Kit is a two component kit: one is the EZ DNA Methylation-Direct™ Kit (D5020) and the other the Universal Methylated Human Standard (D5011) and 2X Zymo Taq™ PreMix (E2003).

DNA Recovery: Typical yields of “bisulfite-converted” DNA are between 80-90%.

Elution: DNA can be eluted with ≥ 10 µl M-Elution Buffer or water.

EZ DNA Methylation-Direct™ Kit technologies are patent pending.

Use of Methylation Specific PCR (MSP) is protected by US Patents 5,786,146 & 6,017,704 & 6,200,756 & 6,265,171 and International Patent WO 97/46705. No license under these patents to use the MSP process is conveyed expressly or by implication to the purchaser by the purchase of this product.

Note - ™ Trademarks of Zymo Research Corporation. This product is for research use only and should only be used by trained professionals. Some reagents included with this kit are irritants. Wear protective gloves and eye protection. Follow the safety guidelines and rules enacted by your research institution or facility.

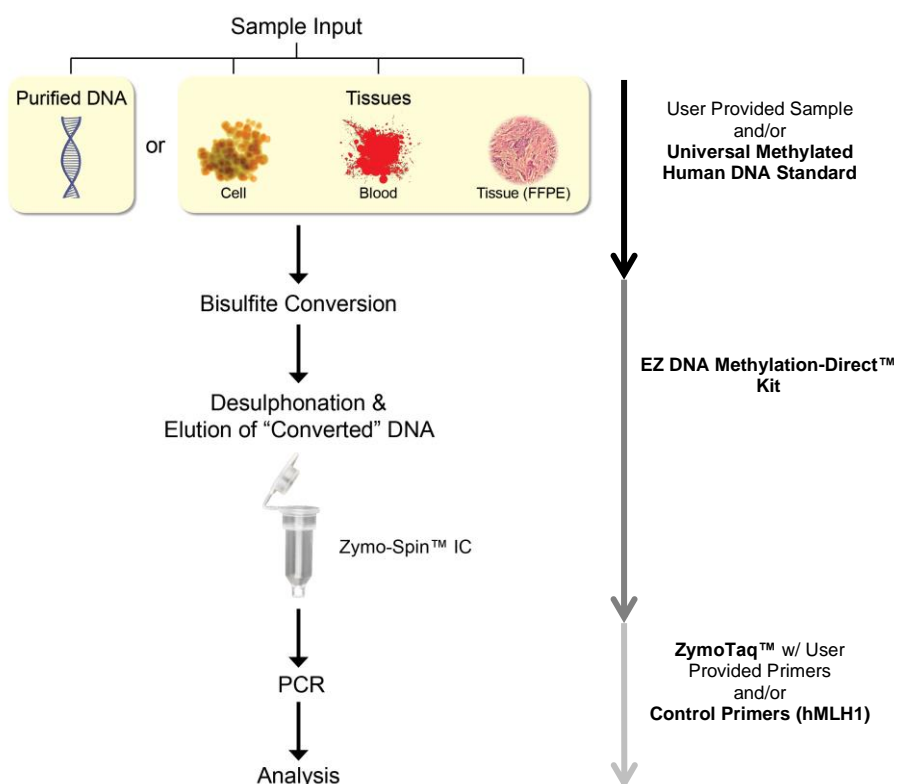
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DNA Methylation and Bisulfite Treatment

DNA methylation has been shown to play key roles in gene imprinting, embryonic development, X-chromosome gene silencing, cell cycle regulation, and is one of the critical factors in regulating epigenetic control of the genome. In most plants and animals, DNA methylation consists of the addition of a methyl group to the fifth carbon position of the cytosine pyrimidine ring creating 5-methylcytosine via a methyltransferase enzyme. The majority of DNA methylation in mammals occurs in the context of 5'-CpG-3' dinucleotides, which can be used as an epigenetic marker in both normal and diseased conditions.

Currently, the most reliable technique for methylation detection has been the bisulfite conversion method. This technique consists of treating DNA with bisulfite, which causes unmethylated cytosines to be “converted” into uracil while methylated cytosines remain unchanged. With the **EZ DNA Methylation-Startup™ Kit**, purified DNA, blood, or tissue samples can be processed directly. After bisulfite modification, the DNA is then amplified by PCR and the resulting PCR products analyzed either by DNA sequencing or by other methods typically used in determining the methylation status of DNA.

Diagrammed Workflow of the EZ DNA Methylation-Startup™ Kit

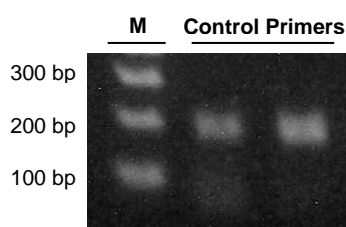


For **Assistance**, please contact Zymo Research Technical Support at 1-888-882-9682 or e-mail tech@zymoresearch.com.

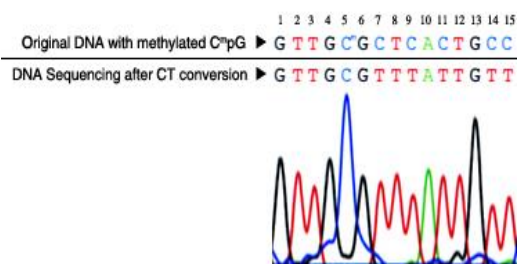
Product Description

Zymo Research's technologies for bisulfite conversion and DNA methylation detection remain the most popular and cited to date. The **EZ DNA Methylation-Startup™ Kit** is designed to include these technologies for the first-time user requiring a consolidated product to perform DNA methylation analysis.

The **EZ DNA Methylation-Startup™ Kit** provides the necessary technologies required for complete bisulfite-conversion of DNA for PCR and methylation analysis. This kit includes bisulfite conversion reagents that allow for direct sampling of blood, cells, and fresh or FFPE tissues without the prerequisite for upstream DNA purification. However, purified DNA can also be bisulfite treated directly. A fully methylated **Universal Methylated Human DNA Standard** is provided together with a special primer set for PCR to control for and assess conversion efficiency. Finally, a unique "hot start" **ZymoTaq™ PreMix** is included for robust amplification of bisulfite-treated DNA.



Efficient PCR amplification of bisulfite-treated DNA for methylation detection. The figure shows amplification of a 182 bp product from bisulfite-treated, **Universal Methylated Human DNA Standard** using hMLH1 primers (1 and 2) and **ZymoTaq™ PreMix**. In duplicate, equal amounts of bisulfite-treated DNA (**EZ DNA Methylation-Direct™ Kit**) were used for each PCR. Products were separated in a 2.0% (w/v) agarose/TAE/EtBr gel. Also evident from the image is the low occurrence of primer dimerization and non-specific product formation.



DNA sequencing results following bisulfite treatment. DNA with methylated C^mpG at nucleotide position #5 was processed using the **EZ 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.

Protocol

For reagent preparation and DNA bisulfite treatment details, please see the **EZ DNA Methylation-Direct™ Kit** instructions. The following protocol uses the supplied **Universal Methylated Human DNA Standard** as an example and should be used as a guideline for your experimental samples.

Section I. Bisulfite Conversion of DNA (please reference the **EZ DNA Methylation-Direct™ Kit** instruction manual as needed)

1. Dilute the DNA in a PCR tube as outlined below...

2 μ l **Universal Methylated Human DNA Standard** (or user
supplied sample - see note below)
18 μ l H_2O
20 μ l total volume

Note: The maximum input volume of DNA is 20 μ l. Alternatively, cells and tissues can be input directly without the prerequisite for DNA purification (reference the **EZ DNA Methylation-Direct™ Kit** protocol).

2. Add 130 μ l of freshly prepared **CT Conversion Reagent** to the DNA in the PCR tube. Mix the sample and then centrifuge briefly to ensure no droplets are retained in the cap or the sides of the tube.
3. Proceed with the **EZ DNA Methylation-Direct™ Kit** protocol through to the DNA elution step (i.e., Step 9).

The eluted DNA will be in Elution Buffer (10 mM Tris-HCl, pH 8.5, 0.1 mM EDTA) and can be used for PCR, microarrays, bisulfite sequencing, or other applications.

(Continued on Next Page...)

The final concentration of ZymoTaq™ DNA Polymerase in the 1X ZymoTaq™ PreMix is 2 U/50 µl.

Protocol (continued)

Section II. PCR Amplification of Bisulfite-Treated DNA

The following PCR conditions have been optimized for amplification of the **Universal Methylated Human DNA Standard** following bisulfite treatment and should be used as a guideline when setting up your own PCR. The control primers (i.e., hMLH1 I and II) are designed to specifically amplify a 182 bp product. See **Appendix I** (page 6) for detailed information regarding the design and use of the **Universal Methylated Human DNA Standard** and **Control Primers**, see **Appendix II** for general information on bisulfite PCR.

1. Set up the following reaction...

12.5 µl	2X ZymoTaq™ PreMix
1 µl	hMLH1 Primer I
1 µl	hMLH1 Primer II
2 µl	Bisulfite-Treated Universal Methylated Human DNA Standard
<u>8.5 µl</u>	<u>ddH₂O</u>
25 µl	Total Volume

Note: The amount of input DNA in the PCR can be increased or decreased as needed. The final concentration of MgCl₂ in the reaction is 1.75 mM. If required, adjust reaction volumes accordingly to optimize the MgCl₂, primer, and/or template concentrations

2. Use the parameters below for amplification of the bisulfite-treated, **Universal Methylated Human DNA Standard**.

<u>Temperature</u>	<u>Time</u>	
95°C	10 minutes	
95°C	30 seconds	} 35-40 cycles
59°C	30 seconds	
72°C	60 seconds	
72°C	7 minutes	
4°C	"hold"	

For amplification of user provided bisulfite-treated DNA, the annealing temperature and extension time should be adjusted according to the primer T_m s and amplicon size, respectively. We recommend using between 35-40 cycles for most templates. Please refer to **Appendix II** for additional PCR and primer design guidelines. The amplified PCR product can be used for gel analysis, TA cloning, restriction endonuclease digestion, microarrays, sequencing, and other downstream molecular applications.

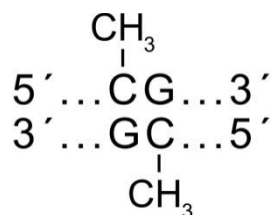
Appendix I: Universal Methylated Human DNA Standard and Control Primers

1. Universal Methylated Human DNA Standard.

Source: DNA derived from a human male. DNA has methylated cytosines (C⁵) at all CG dinucleotide positions.

Concentration: 250 ng/μl in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0)

Methylation Site:



2. Control Primers

Two hMLH1 Control Primers are supplied at a concentration of 20 μM (each) in 20 μl TE buffer. The expected PCR amplicon is **182 bp**, corresponding to nucleotide positions **804 - 986** of the hMLH1 DNA sequence that includes the regions spanned by the primers (see below).

hMLH1 Primer I (sense)

5'-GGAGTGAAGGAGGTTACGGGTAAGT-3'

hMLH1 Primer II (antisense)

5'-AAAAACGATAAAACCCTATACCTAATCTATC-3'

Original sequence of the human MLH1 DNA fragment for bisulfite treatment and PCR amplification (sense strand 5' to 3'). All cytosines in CG dinucleotides (bold) are methylated. Numbers correlate to the nucleotides from the human MLH1 DNA 5' flanking region (GenBank Accession #U83845). Observed CpG/ Expected CpG is > 1 with a 68% total GC content.

```

781 -----
841 gaCGctccac cagggcCGCG CGctCGcCGt cCGccacata cCGctCGtag tattCGtgct
901 cagcctCGta gtggCGcctg aCGtCGCGtt CGCGggtagc taCGatgagg CGgCGacaga
961 ccaggcacag ggccccatCG ccctc

```

Expected Sequence of PCR amplified product following bisulfite conversion (sense strand 5' to 3').

```

781 -----
841 gaCGttttat tagggtCGCG CGttCGtCGt tCGttatata tCGttCGtag tattCGtgct
901 tagtttCGta gtggCGtttg aCGtCGCGtt CGCGggtagt taCGatgagg CGgCGataga
961 ttagtatag ggttttatCG ttttt

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Note: Methylated "C" is underlined in the examples.

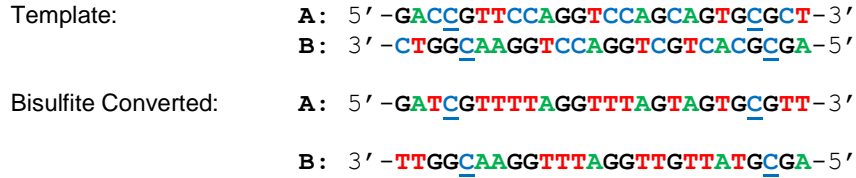
Note: Following bisulfite conversion, the strands are no longer complementary.

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.

Appendix II: 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.



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.



Zymo Research provides primer design assistance with its [Bisulfite Primer Seeker Program](http://www.zymoresearch.com/tools/bisulfite-primer-seeker), available at: www.zymoresearch.com/tools/bisulfite-primer-seeker

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.

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 $A_{260} = 1.0$ when determining the concentration of the recovered bisulfite-treated DNA.

Epigenetics Products From Zymo Research

Product	Description	Kit Size	Cat No. (Format)
Bisulfite Kits for DNA Methylation Detection			
EZ DNA Methylation™ Kit	For the conversion of unmethylated cytosines in DNA to uracil via the <u>chemical-denaturation</u> of DNA and a specially designed CT Conversion Reagent. <i>Fast-Spin</i> technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5001 (spin column) D5002 (spin column) D5003 (shallow-well plate) D5004 (deep-well plate) D5040 (magnetic bead) D5041 (magnetic bead)
EZ DNA Methylation-Gold™ Kit	For the fast (3 hr.) conversion of unmethylated cytosines in DNA to uracil via <u>heat/chemical-denaturation</u> of DNA and a specially designed CT Conversion Reagent. <i>Fast-Spin</i> technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5005 (spin column) D5006 (spin column) D5007 (shallow-well plate) D5008 (deep-well plate) D5042 (magnetic bead) D5043 (magnetic bead)
EZ DNA Methylation-Direct™ Kit	Features simple and reliable DNA bisulfite conversion directly from blood, tissue (FFPE/LCM), and cells without the prerequisite for DNA purification in as little as 4-6 hrs. The increased sensitivity of this kit makes it possible to amplify bisulfite converted DNA from as few as 10 cells or 50 pg DNA. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5020 (spin column) D5021 (spin column) D5022 (shallow-well plate) D5023 (deep-well plate) D5044 (magnetic bead) D5045 (magnetic bead)
EZ DNA Methylation-Lightning™ Kit	Complete bisulfite conversion in about an hour using a unique liquid format conversion reagent that requires no preparation. <i>Fast-Spin</i> technology ensures ultra-pure, converted DNA for subsequent DNA methylation analysis. Magnetic bead format for adaptation to automated liquid handling platforms.	50 Rxns. 200 Rxns. 2x96 Rxns. 2x96 Rxns. 4x96 Rxns. 8x96 Rxns.	D5030 (spin column) D5031 (spin column) D5032 (shallow-well plate) D5033 (deep-well plate) D5046 (magnetic bead) D5047 (magnetic bead)
EZ DNA Methylation-Startup™ Kit	Designed for the first time user requiring a consolidated product to perform DNA methylation analysis. Includes technologies for sample processing, bisulfite treatment of DNA, and PCR amplification of "converted" DNA for methylation analysis.	1 Kit	D5024
Methylated DNA Standards			
Universal Methylated Human DNA Standard	Human (male) genomic DNA having all CpG sites methylated. To be used for the evaluation of bisulfite-mediated conversion of DNA. Supplied with a control primer set.	1 set	D5011
Universal Methylated Mouse DNA Standard	Mouse (male) DNA having all CpG sites methylated. To be used for the evaluation of bisulfite-mediated conversion of DNA. Supplied with a control primer set.	1 set	D5012
Other...			
ChIP DNA Clean & Concentrator™	Clean and concentrate DNA from any reaction or "crude" preparation in 2 min. A 6 µl minimum elution volume allows for highly concentrated DNA. Designed for samples containing up to 5 µg of DNA.	50 Preps. 50 Preps.	D5201 (uncapped column) D5205 (capped column)
Genomic DNA Clean & Concentrator™	Genomic DNA clean-up in minutes. Unique spin column technology for recovery of ultra-pure large-sized DNA (100 bp to ≥200 kb) DNA from any impure preparation (e.g., Proteinase K digestion).	25 Preps. 100 Preps.	D4010 D4011
ZymoTaq™ DNA Polymerase	ZymoTaq™ "hot start" DNA Polymerase is specifically designed for the amplification of "difficult" DNA templates including: bisulfite-treated DNA for methylation detection. The product generates specific amplicons with little or no by-product formation. Available either as a single buffer premix or as a polymerase system with components provided separately.	50 Rxns. 200 Rxns. 50 Rxns. 200 Rxns.	E2001 (system) E2002 (system) E2003 (premix) E2004 (premix)
Methylated-DNA IP Kit	IP with a highly specific anti-5-methylcytosine monoclonal antibody. Designed for the enrichment of 5-methylcytosine-containing DNA from any pool of fragmented genomic DNA for use in genome-wide methylation analysis.	10 Rxns.	D5101

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