invitrogen™

MethylCode™ Bisulfite Conversion Kit

Catalog no. MECOV-50

Version A
6 March 2007
25-1015

User Manual

Table of Contents

Kit Contents and Storage

Shipping and Storage

The MethylCode™ Bisulfite Conversion Kit is shipped at room temperature. Store all components at room temperature.

Kit Components

Components are provided for 50 reactions.

Component	Amount
CT Conversion Reagent (prepare each tube before use as described on page 5)	5 tubes
Dilution Buffer	1.5 ml
Resuspension Buffer	500 µl
Binding Buffer	30 ml
Wash Buffer (add ethanol before first use as described below)	6 ml
Desulphonation Buffer	10 ml
Elution Buffer	1 ml
Spin Columns	50
Collection Tubes	50

Preparing the Wash Buffer with Ethanol

Add **24 ml** of 100% ethanol directly to the 6 ml of Wash Buffer provided in the kit to make the final Wash Buffer with ethanol. We recommend marking the label on the bottle to indicate that the ethanol has been added.

Store the Wash Buffer with ethanol at room temperature.

Additional Products

Additional Products

Invitrogen has additional reagents for use with this kit. To order, visit our online catalog at www.invitrogen.com or contact Technical Support (see page 10). Ordering information is provided below.

Product	Quantity	Catalog no.
PureLink™ Genomic DNA Purification Kit	50 preps	K1810-01
PureLink™ 96 Plasmid Purification System	4 × 96 preps	12263-018
E-Gel® 1.2% Starter Pak	6 gels and base	G6000-01
Taq DNA Polymerase, native (5 U/µl)	100 units 500 units	18038-018 18038-042
Taq DNA Polymerase, recombinant (5 U/µl)	100 units 500 units	10342-053 10342-020
Platinum® *Taq* DNA Polymerase	100 rxns 250 rxns 500 rxns	10966-018 10966-026 10966-034
Platinum® *Taq* DNA Polymerase High Fidelity	100 rxns 500 rxns	11304-011 11304-029
Platinum® *Pfx* DNA Polymerase	100 rxns 250 rxns	11708-013 11708-021
Pfx50™ DNA Polymerase	100 rxns	12355-012

Overview

Introduction

The ability to quantify and detect DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, and genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to quantify DNA methylation, including high-performance capillary electrophoresis and methylation-sensitive arbitrarily primed PCR (Fraga *et al.*, 2000; Gonzalgo *et al.*, 1997). Currently, the most commonly used technique has been the bisulfite method (Frommer *et al.*, 1992). This method consists of treating DNA with bisulfite, which causes unmethylated cytosines to be converted into uracil while methylated cytosines remain unchanged. The bisulfite-modified DNA is then amplified by PCR and the resulting PCR products are either analyzed by DNA sequencing or by restriction endonuclease digestion. The methylation profile of the DNA segment is determined by comparing the sequence of the bisulfite-treated DNA to that of the untreated DNA.

The MethylCode™ Bisulfite Conversion Kit integrates the DNA denaturation and bisulfite conversion processes into one step, by using temperature denaturation to replace chemical denaturation with sodium hydroxide. Following bisulfite conversion, the DNA is recovered using a streamlined in-column desulphonation method.

The recovered DNA is ready for PCR amplification and downstream analyses, including restriction endonuclease digestion, sequencing, microarrays, etc.

Advantages of the System

- Integrates the DNA denaturation and bisulfite conversion processes into one convenient step
- Provides complete conversion of unmethylated cytosines
- Innovative in-column desulphonation technology eliminates cumbersome DNA precipitation steps
- Minimizes template degradation and loss of DNA during treatment and clean-up
- Recovered DNA is ready for PCR amplification and downstream analysis

Continued on next page

Overview, continued

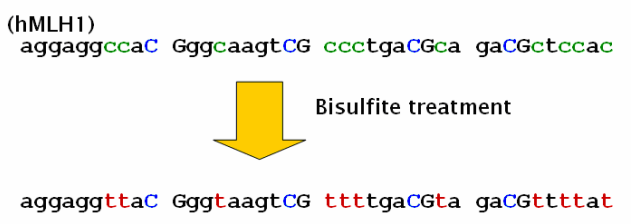
DNA Methylation

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 their own restriction enzymes that are designed to eliminate foreign DNA. In higher eukaryotes DNA methylation acts as another method for the regulation of gene expression (Costello & Plass, 2001).

Aberrant methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis (Stirzaker *et al.*, 1997). 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 (Adams, 1995). The majority of DNA methylation in mammals occurs in 5′-CpG-3′ dinucleotides, but other methylation patterns also 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 20 percent that remain unmethylated are within promoters or in the first exons of genes.

Example of Bisulfite Conversion



DNA sequencing before and after bisulfite conversion reveals that methylated cytosines (in caps) remain intact while unmethylated cytosines (lower case) are completely converted to uracil and detected as thymine following PCR.

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Overview, continued

Materials Supplied by the User

In addition to the kit components, the following materials are required to perform the procedures in this manual:

- Vortex mixer
- Microcentrifuge
- Thermocycler
- Thin-walled PCR tubes
- 1.5-ml microcentrifuge tubes
- 100% ethanol (for preparing the Wash Buffer; see page v)

Methods

Starting Material

Amount of Starting Material

The amount of DNA per treatment can range from 500 pg to 2 µg. An optimal amount is 200–500 ng. Starting material may be in water or in a buffer such as TE.

Note

Larger amounts of DNA (500 ng–2 µg) may result in incomplete bisulfite conversion of some GC-rich regions.

Isolating DNA

You can isolate DNA using your method of choice. The PureLink™ Genomic DNA Purification Kit (K1810-01) is a complete kit for the isolation of genomic DNA. See page vi for ordering information. A wide range of ChargeSwitch® Genomic DNA purification kits are also available from Invitrogen.

DNA Treatment

The DNA may be either intact or treated by enzymatic digestion or sonication.

General Handling of DNA

When handling DNA, use sterile conditions to ensure that no DNases are introduced. All equipment that comes into contact with DNA should be sterile, including pipette tips, microcentrifuge tubes, snap-cap polypropylene tubes, and pipettes. Be sure pipettor barrels are clean and treated with ethanol.

Checking DNA Quantity and Quality

Genomic DNA may be run on an agarose gel to check for quantity and quality. Bufferless E-Gel® Pre-cast Agarose Gels are available from Invitrogen for fast and easy electrophoresis. See page vi for ordering information.

Storing DNA

Isolated genomic DNA may be stored at +4°C until use. Note that storage in TE Buffer is recommended for greater stability.

Preparing the CT Conversion Reagent

Preparing the Reagent

CT Conversion Reagent is supplied as a dry mixture. Prepare each tube as follows:

1. Add 900 µl* of sterile distilled water, 50 µl of Resuspension Buffer, and 300 µl of Dilution Buffer directly to one tube of CT Conversion Reagent.

   ***Note:** If you are treating DNA sample volumes >20 µl, you need to *decrease* the amount of water used to prepare the reagent; see below for details.
2. Mix by shaking or intermittent brief vortexing for 10 minutes. Trace amounts of undissolved material may remain, which is normal. (The reagent is saturated in solution.)
3. Keep protected from light at room temperature (20°–30°C) until use.

Each tube provides enough reagent for 10 DNA treatments. For best results, use the reagent immediately after preparation. For storage, see below.

Storing Prepared Reagent

CT Conversion Reagent that has been prepared in solution may be stored up to 1 week at –20°C. Thaw at room temperature and mix for 2 minutes by rotating or vortexing before use.

Preparing Reagent for DNA Volumes >20 µl

If you are treating DNA volumes greater than 20 µl, *decrease* the amount of water used to prepare the CT Conversion Reagent by 100 µl for each 10 µl *increase* in DNA sample volume (above 20 µl).

Example: For a DNA sample volume of 40 µl, add 700 µl of water to prepare the CT Conversion Reagent.

Note

CT Conversion Reagent is light sensitive. Minimize exposure to light.

Bisulfite Conversion Protocol

Before Starting

In addition to the kit components, the following materials are supplied by the user:

- DNA
- Vortex mixer
- Microcentrifuge
- Thermocycler
- Thin-walled PCR tubes
- 1.5-ml microcentrifuge tubes

Bisulfite Conversion Procedure

The amount of DNA per treatment can range from 500 pg to 2 µg. An optimal amount is 200–500 ng.

1. If necessary, add sterile distilled water to the DNA sample to bring the volume up to 20 µl.

   Note: For DNA volumes greater than 20 µl, see the special instructions for preparing the CT Conversion Reagent on page 5. In the next step, add the specially prepared CT Conversion Reagent to the DNA to a total volume of 150 µl (DNA + Reagent).

2. In a PCR tube, add 130 µl of CT Conversion Reagent (prepared as on page 5) to the 20-µl DNA sample. Mix by flicking the tube or pipetting up and down.

3. Place the tube in a thermal cycler and run the following program:

   98°C for 10 minutes (DNA denaturation)

   64°C for 2.5 hours (Bisulfite conversion)

   4°C storage for up to 20 hours

   Note: Alternative cycling programs for longer DNA templates are provided on page 8.

4. Place a Spin Column in a Collection Tube and add 600 µl of Binding Buffer to the column.

5. Add the sample from Step 3 to the Binding Buffer in the column. Close the cap and mix by inverting several times.

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

Procedure continued on next page

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Bisulfite Conversion Protocol, continued

Bisulfite Conversion Procedure, continued

Procedure continued from previous page

7. Add 100 µl of Wash Buffer prepared with ethanol (as described on page v) to the column, and centrifuge at full speed for 30 seconds. Discard the flow-through.
8. Add 200 µl of Desulphonation Buffer to the column and let the column stand at room temperature for 15–20 minutes.
9. Centrifuge at full speed for 30 seconds. Discard the flow-through.
10. Add 200 µl of Wash Buffer with ethanol to the column and centrifuge at full speed for 30 seconds. Discard the flow-through.
11. Repeat the wash in Step 10 one more time, then transfer the spin column to a new, clean 1.5-ml microcentrifuge tube.
12. Add 10 µl of Elution Buffer directly to the column matrix. Centrifuge at full speed for 30 seconds to elute the DNA.

The DNA is ready for immediate analysis, or can be stored at or below –20°C. We recommend using 2–4 µl of the DNA for each PCR.

Appendix

Optimization and PCR Guidelines

Alternative Thermal Cycling Programs

The thermal cycling program provided in the Bisulfite Conversion Procedure, Step 3, page 6, will generate consistent results for both easy and difficult-to-convert DNA templates, including GC-rich templates.

The alternative programs below may yield better results in PCR amplification of longer DNA fragments. Note that these programs may result in incomplete cytosine-to-uracil conversion of DNA templates with >80% GC composition.

Alternative 1:

1. 98°C for 10 minutes
2. 53°C for 30 minutes
3. 8 cycles of:
   53°C for 6 minutes
   37°C for 30 minutes
4. 4°C storage

Alternative 2:

1. 98°C for 10 minutes
2. 53°C for 4 hours
3. 4°C storage

Primer Design

For PCR amplification of the bisulfite-converted DNA, we recommend using primers that are 24–26 bases in length. For human and mouse DNAs, all unmethylated C's (*i.e.*, non-CpG) will be converted into uracils during the bisulfite treatment. Therefore, these C's should be treated as T's for primer design purposes. For example, for the sequence 5´-AACCTTACAGGCAC-3´, the corresponding primer should be 5´-AA**T**T**T**TA**T**AGG**T**A**T**-3´.

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Optimization and PCR Guidelines, continued

PCR Guidelines

Usually, 35–40 PCR cycles are required for successful amplification of bisulfite-converted DNA. The optimal amplicon size is between 150–500 bp; however, larger amplicons (up to 1 kb) can be generated with optimization of the bisulfite reaction and PCR.

We have found that annealing temperatures between 55°C and 60°C work well. Since most cytosine residues are converted into uracil, the bisulfite-treated DNA usually is AT rich, with low GC composition. Thus, in most instances, it will be necessary to reduce the annealing temperature during PCR.

DNA Polymerases

For PCR, Invitrogen offers the following DNA polymerases (for ordering information, see page vi).

- ***Taq* DNA Polymerase** for standard PCR
- **Platinum® *Taq* DNA Polymerase** provides automatic hot-start conditions for increased specificity up to 4 kb
- **Platinum® *Taq* DNA Polymerase High Fidelity** provides increased yield and high fidelity for targets up to 15 kb
- **Platinum® *Pfx* DNA Polymerase** provides very high fidelity (25X compared to *Taq* DNA Polymerase) for targets up to 12 kb
- ***Pfx50*™ DNA Polymerase** provides maximum fidelity (50X compared to *Taq* DNA Polymerase) for targets up to 4 kb

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, FAQs, formulations, citations, handbooks, MSDSs, CofAs, etc.
- Complete technical service contact information
- Access to the Invitrogen Online Catalog
- Additional product information and special offers

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MSDSs (Material Safety Data Sheets) are available on our website at www.invitrogen.com/msds.

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The Certificate of Analysis (CofA) for your product is available on our website at www.invitrogen.com/cofa.

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