EPIGENETICS

EpiMark®
Bisulfite Conversion Kit

Instruction Manual

NEB #E3318S
48 reactions

NEW ENGLAND BioLabs® Inc.
enabling technologies in the life sciences

Safety Information:

The EpiMark Bisulfite Conversion Kit is intended for research purposes only and not for human or drug use. This kit is not designed for diagnostic purposes. Ensure that lab coats and gloves are worn when working with this kit. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Bisulfite Mix contains sodium metabisulfite, which is hazardous in case of skin contact (irritant), of ingestion, of inhalation.

The binding buffer contains sodium perchlorate, and should be handled with care. Sodium perchlorate is explosive when mixed with combustible material, harmful by inhalation and if swallowed, irritating to eyes, respiratory system and skin.

The Solubilization Buffer and Desulfonation Reaction Buffer contain sodium hydroxide, which is strongly corrosive and a powerful irritant.

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Kit Components:

Each EpiMark Bisulfite Conversion Kit contains sufficient reagents to perform the bisulfite conversion reaction of 48 samples. Store at room temperature (15–20°C). However, for long-term storage (longer than 4 weeks) Desulphonation Reaction Buffer concentrate should be stored at 2–8°C. Prepared Bisulfite Mix can be stored at –20°C for up to six months.

Sodium metabisulfite (each aliquot is sufficient for 8 reactions) 6

Solubilization Buffer ..... 2 x 1 ml

Desulphonation Reaction Buffer (concentrate) ..... 3 ml

EpiMark spin columns with 2 ml collection tubes 50

Binding Buffer ..... 30 ml

Wash Buffer (concentrate) ..... 2 x 10 ml

Elution Buffer ..... 4 ml

Required Materials Not Included

Heat block or water bath (requiring temperatures of 65°C and 92°C)

96–100% molecular biology grade ethanol

PCR Thermal Cycler

0.2 ml strip tubes and caps

1.5 ml reaction tubes

Nuclease-free Water

Pipettors and pipette tips (for minimization of cross contamination, use aerosol barrier tips)

Introduction:

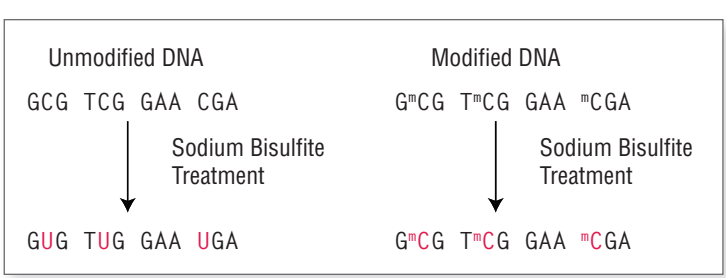
Genomic DNA from many organisms has modified nucleotides. In the mammalian genome, the modified base is predominately 5-methylcytosine (5-mC), which is involved in gene expression regulation, including selective inactivation of one X chromosome in females of mammalian species (1,2). Symmetrical CpG DNA methylation is heritable, but also reversible. This change of methylation status results in an enormous number of combinations of epigenetic states that can regulate gene expression. In mammalian cells, DNA methylation mainly occurs in CpG dinucleotides and is carried out by two methyltransferase enzymatic activities, namely, maintenance methylation and de novo methylation. The maintenance methyltransferase, DNMT1, is involved in the DNA methylation after every cellular DNA replication cycle. DNMT3a and DNMT3b are the *de novo* methyltransferases that are active in early development.

Method Overview:

Many tools can be used to determine the methylaton status of DNA, including antibodies, methyl binding proteins, methylation-dependent restriction enzymes, and most recently next generation sequencing. However, the most commonly used technique to date is sodium bisulfite conversion, the "gold standard" for methylation analysis (3). Incubation of the target DNA with sodium bisulfite results in conversion of all unmodified cytosines to uracils leaving the modified bases (5-mC or 5-hmC) intact. (see Figure 1). The most critical step in methylation analysis using bisulfite conversion is the complete conversion of unmodified cytosines. This is achieved by alternating cycles of thermal denaturation with incubation reactions. The protocol optimized for this kit is simple and gives consistent results.

This kit provides materials for 48 reactions. For optimal results, 50 ng–2 µg DNA is recommended.

Figure 1. Overview of Bisulfite Conversion Reaction



Methylated cytosines are protected and remain unchanged, while unmethylated cytosines are deaminated to uracil after treatment with sodium bisulfite.

Protocols:

Reagent Preparation

Add 40 ml of ethanol (96–100%) to Wash Buffer concentrate and store at room temperature (18–25°C). Invert the bottle several times before starting sample clean up procedure.

Add 27 ml of ethanol (96–100%) to Desulphonation Reaction Buffer concentrate. Store up to 4 weeks at room temperature (18–25°C); for longer than 4 weeks store at 2–8°C. Invert the bottle several times before starting each desulphonation reaction.

Prepare the bisulfite mix by adding 650 µl of nuclease-free water and 250 µl of Solubilization Buffer to solid sodium metabisulfite in the tube provided. Vortex until the bisulfite mix is completely dissolved and clear, usually between 2–5 minutes. If some white particles are still present, heat the solution to 55°–60°C for a few minutes and vortex briefly. Each vial of bisulfite mix is sufficient for 8 reactions. Excess bisulfite mix, that is not used immediately, can be stored frozen for up to 6 months.

Step I – Bisulfite Conversion Reaction

Combine the following in a 0.2 ml PCR tube:

REAGENT	VOLUMES PER REACTION
Genomic DNA	10.0 µl (50 ng–2 µg)
Bisulfite Mix	130.0 µl
Total Reaction Volume	140.0 µl

Mix gently by pipetting.

Step II – Bisulfite Conversion Reaction

Transfer reaction tube to a thermocycler and begin cycling:

CYCLE STEP	TEMP	TIME
Denaturation	95°C	5 Minutes
Incubation	65°C	30 Minutes
Denaturation	95°C	5 Minutes
Incubation	65°C	60 Minutes
Denaturation	95°C	5 Minutes
Incubation	65°C	90 Minutes
Hold	18–20°C	up to 12 hours

Step III – Desulphonation Reaction and Sample Clean Up

1. After completion of the conversion reaction, transfer the individual reactions into 1.5 ml microcentrifuge tubes, add 550 µl of DNA Binding Buffer and mix briefly.
2. Load the entire sample onto an EpiMark spin column with 2 ml collection tube attached. Centrifuge the columns at 15,000 x g for 1 minute and discard the flow through.
3. Add 500 µl of Wash Buffer, centrifuge the columns at 15,000 x g for 1 minute and discard the flow through.
4. Add 500 µl of Desulphonation Reaction Buffer to each column and incubate at room temperature (18–20°C) for 15 minutes. Close the lids of the spin columns during incubation.
5. Centrifuge the columns at 15,000 x g for 1 minute and discard the flow through.
6. Add 500 µl of Wash Buffer, centrifuge the columns at 15,000 x g for 1 minute and discard the flow through. Repeat wash step.
7. Centrifuge the columns at 15,000 x g for 1 minute to remove any residual Wash Buffer from the spin columns.
8. Place the spin columns into sterile 1.5 ml microcentrifuge tubes (not provided). Add 20 µl of Elution Buffer, incubate for 1 minute and centrifuge the columns at 15,000 x g for 1 minute. Add an additional 20 µl of Elution Buffer and spin again for 30 seconds at 15,000 x g.

The DNA is ready for methylation analysis by PCR. We recommend using 1–6 µl of DNA for each PCR reaction.

End-point PCR:

1. Add the following components to a 0.2 ml PCR reaction tube:

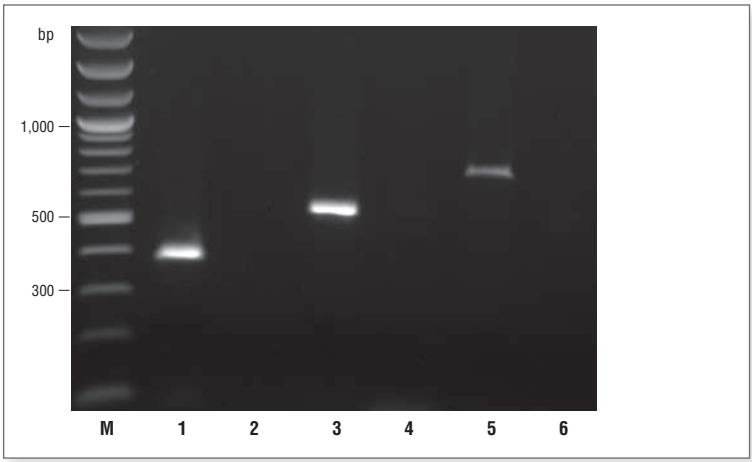
PCR COMPONENT	25 µl REACTION	50 µl REACTION	FINAL CONCENTRATION
5X EpiMark Hot Start *Taq* Reaction Buffer	5 µl	10 µl	1X
10 mM dNTPs	0. 5 µl	1. µl	200 µM
10 µM Forward Primer	0.5 µl	1 µl	0.2 µM
10 µM Reverse Primer	0.5 µl	1 µl	0.2 µM
Template DNA	1–6 µl	1–6 µl	up to 1 µg
EpiMark Hot Start *Taq* DNA Polymerase	0.125 µl	0.25 µl	1.25 units/50 µl PCR reaction
Nuclease-free water	Up to 25 µl	Up to 50 µl	

2. Gently mix the reaction. If necessary, collect all liquid to the bottom of the tube by a quick spin. Overlay the sample with mineral oil if using a thermocycler without a heated lid.
3. Transfer PCR tubes from ice to a thermocycler with the block preheated to 94°C and start the cycling program.

Cycling Protocol:

CYCLE STEP	TEMP	TIME
Initial Denaturation	95°C	30 Seconds
35–40 cycles	95°C	15 Seconds
	45–55°C	30 Seconds
	68°C	1 Minute/kb
Final Extension	68°C	5 Minutes

Figure 2. Performance evaluation of the EpiMark Bisulfite Conversion Kit.



One µg of genomic DNA was bisulfite treated using the Epimark Bisulfite Conversion Kit, and 2 µl of eluted DNA was analyzed by end-point PCR. 388, 544, and 731 bp amplicons were amplified with primer pairs for bisulfite converted DNA (lanes 1, 3, and 5), or with primer pairs for unconverted DNA (lanes 2, 4, and 6). Epimark Hot Start Taq DNA Polymerase was used for amplification. Lanes 2, 4, and 6 clearly show no amplification product, indicating complete DNA conversion. Marker M is the 2-Log DNA Ladder (NEB #N3200).

Figure 3. Conversion of a methylated DNA fragment.

```
GAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAG
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
------C---T------TT------T--T-----T----T-----
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50 nanograms of methylated plasmid DNA was bisulfite converted and a 146 bp DNA fragment was amplified using Epimark Hot Start Taq DNA Polymerase (NEB #M0490). Amplicons were cloned, and individual clones were sequenced to determine the methylation status. Sequence aligment of 10 clones is shown to demonstrate conversion of unmethylated cytosine to thymine, while the unconverted methylated cytosine remains intact. The known methylated cytosines at the CpG site shown in red, converted cytosines marked as T, and rest of the sequence is shown as a dashed line.

Frequently Asked Questions (FAQ's)

How should I store my bisulfite converted DNA after it is eluted from the column?

The converted DNA is stable for one day at room temperature, one week at 4°C, and two to four months at –20°C.

Does my sample need to be free of RNA for bisulfite conversion?

No, residual RNA will not interfere with the conversion reaction.

No PCR product is detected when I amplify more than 300 bp amplicon.

There is a possibility that sample DNA was degraded before bisulfite conversion reaction. Ensure that sample DNA is stored correctly.

Do I need extra purification of my mammalian DNA prep in order to use it in a bisulfite conversion reaction?

No, all commercially available genomic DNA purification kits give satisfactory results.

What is the shelf life of the reagents supplied?

We guarantee that all reagents will remain active for at least 12 months when stored at room temperature.

Which DNA polymerase do you recommend for amplification of converted DNA?

EpiMark Hot Start *Taq* DNA Polymerase is a very good choice (NEB #M0490), because no initial activation time is required and the buffer composition is optimized for AT-rich PCR. Hot start DNA polymerases requiring > 2 minutes for activation are not recommended; because the DNA after bisulfite reaction is degraded, heating for > 2 extra minutes at 95°C will cause severe degradation.

My thermocycler does not allow to set up the reaction volume to 140 µl, what I should do?

Set the thermocycler to the largest volume available, usually 100 µl setting works very well.

References:

1. Bestor, T.H., Verdine, G.L. (1994) *Curr. Opin. Cell Biol.* 6, 380–389.
2. Ooi, S.K., O'Donnell A.H., Bestor T.H. (2009) *J. Cell Sci.* 122, 2787–2791.
3. Frommer, M., et. al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 1827–1831.
4. Kumaki, Y., Oda, M. and Okano, M. (2008) *Nucleic Acids Res*., 36, W170–175.

Ordering Information

PRODUCT	NEB #	SIZE
EpiMark Bisulfite Conversion Kit	E3318S	48 reactions
COMPANION PRODUCTS		
EpiMark Hot Start *Taq* DNA Polymerase	M0490S/L	100/500 reactions
NIH 3T3 Mouse Genomic DNA	N4004S	15 µg
HeLa Genomic DNA	N4006S	15 µg

USA
New England Biolabs, Inc.
240 County Road
Ipswich, MA 01938-2723
Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227
Toll Free: (USA Tech) 1-800-632-7799
Fax: (978) 921-1350
e-mail: info@neb.com
www.neb.com

Canada
New England Biolabs, Ltd.
Telephone: (905) 837-2234
Toll Free: 1-800-387-1095
Fax: (905) 837-2994
Fax Toll Free: 1-800-563-3789
e-mail: info@ca.neb.com

China, People's Republic
New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com

France
New England Biolabs France
Free Call: 0800/100 632
Free Fax: 0800/100 610
e-mail: info@fr.neb.com

Germany
New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Fax +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info@de.neb.com

Japan
New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax +81 (0)3 5669 6192
e-mail: info@neb-japan.com

United Kingdom
New England Biolabs (UK), Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info@uk.neb.com

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