Bisulfite Conversion Kit

(version A1)

Catalog No. 55016

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, California 92008, USA
Toll free: 877 222 9543
Telephone: 760 431 1263
Fax: 760 431 1351

Active Motif Europe
Avenue Reine Astrid, 92
B-1310 La Hulpe, Belgium
UK Free Phone: 0800 169 31 47
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733

TABLE OF CONTENTS **Page**

Overview

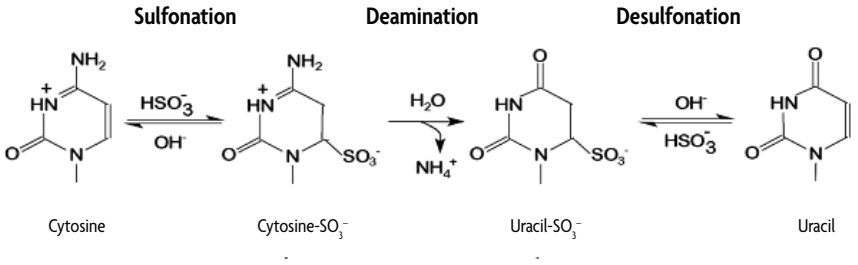
For the investigation of DNA methylation patterns, bisulfite conversion and subsequent DNA sequencing is the gold standard because it provides detailed information on the methylation pattern of individual DNA molecules at single-base-pair resolution. Bisulfite conversion is based on the deamination of cytosine residues into uracils in the presence of sodium bisulfite. Since methylated cytosine residues (both 5-mC and 5-hmC) are not converted under these conditions, the original methylation state of the DNA can be determined. Converted DNA can be analyzed by PCR amplification followed by DNA sequencing (gene-specific or genome-wide), array-based methods, or other downstream applications.

Active Motif's Bisulfite Conversion Kit simplifies analysis of DNA methylation by providing optimized conversion reagents, an easy-to-use protocol and a positive control conversion-specific PCR primer pair that is specific for bisulfite-converted human or mouse DNA. Because the primer pair produces a PCR product only if conversion has occurred, you can confirm the procedure worked before starting sequencing or other analysis methods.

In the Bisulfite Conversion Kit method, DNA of interest is rapidly heat denatured in a thermocycler in the presence of the bisulfite conversion reagent and a DNA denaturant. The conversion reaction can be performed is as little as 1.5 hours. After DNA conversion, the sample is added to the included DNA purification columns, and a simple, on-column desulfonation is performed. Ready-to-use converted DNA is then eluted from the columns. For your convenience, the included positive control conversion-specific PCR primer pair can be used to assess the success of the bisulfite conversion before DNA sequencing because the included primers only anneal to converted human or mouse DNA.

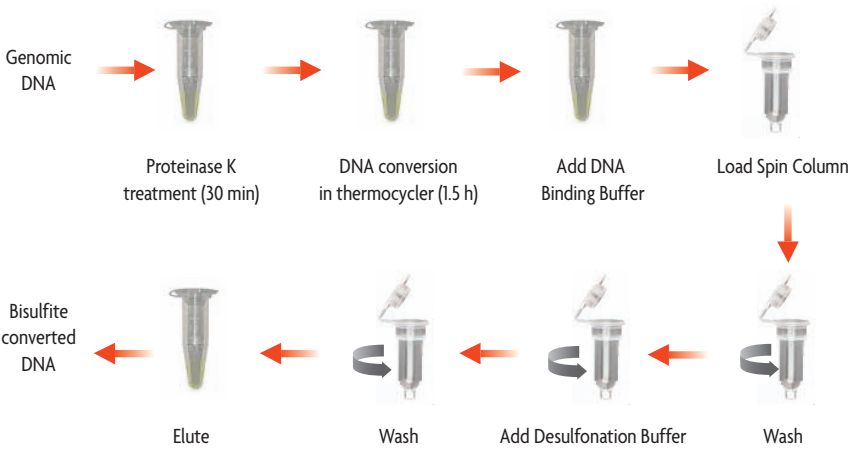
product	format	catalog no.
Bisulfite Conversion Kit	50 rxns	55016

Flow Chart of Process



Schematic of the bisulfite conversion reaction of unmethylated cytosine to uracil.

The first step in the bisulfite conversion reaction is sulfonation, where sodium bisulfite is added to the double bond of cytosine for form a cytosine-bisulfite derivative. This reaction is reversible, with the forward reaction being favored by high temperature and low pH. The second step is an irreversible hydrolytic deamination of the cytosine-bisulfite derivative that results in a uracil-bisulfite derivative. This reaction is also favored by low pH. The final step involves desulfonation of the uracil-bisulfite to uracil under high pH conditions. Only unmethylated cytosines are susceptible to the bisulfite reaction. Methylated (5-mC and 5-hmC) cytosines do not undergo conversion.



Flow chart of the Bisulfite Conversion Kit.

In the Bisulfite Conversion Kit, genomic DNA of interest is proteinase K treated, then rapidly heat denatured in a thermocycler in the presence of the bisulfite conversion reagent and DNA denaturant. Following a 1.5 hour DNA conversion reaction, the sample is added to the included DNA purification columns, and a simple on-column desulfonation is performed. DNA is then eluted from the column and is ready for use in PCR.

Introduction

DNA methylation is an essential epigenetic modification that plays a key role in transcriptional regulation and assures the proper regulation of gene expression and stable gene silencing in normal cells. DNA methylation is the covalent addition of a methyl group to the fifth position of the cytosine pyrimidine ring in DNA and usually occurs within CpG dinucleotides, although in some cases the DNA methylation can also be found in a non-CpG context[1, 2]. CpG dinucleotides are concentrated in large clusters called CpG islands, which are often positioned in the promoter regions of many genes and are usually unmethylated[3]. It is estimated that the human genome contains around 29,000 CpG islands and that almost 60% of all human genes are associated with CpG islands[4]. Aberrant cytosine methylation is associated with silencing of tumor suppressor genes[5] and plays a decisive role in the development of many cancers[6-14]. Alterations in the methylation status of DNA are promising candidates for highly specific and sensitive indicators of cancer diagnosis and prognosis[15-17]. Apart from carcinogenesis, DNA methylation is crucial for a variety of processes, such as genomic imprinting, X-chromosome inactivation, and suppression of repetitive elements[4, 18]. Thus profiling DNA methylation across the genome is vital to understanding the influence of epigenetics.

The bisulfite reaction was first described in the early 1970s[19] and was used by Frommer *et al*.[20], and Clark *et al*.[21] to distinguish between cytosine and 5-methylcytosine (5-mC) in DNA. It is now known that both 5-methylcytosine and 5-hydroxymethylcytosine (5-hmC) remain unchanged during conversion and therefore this method cannot be used to distinguish between the different methyl modifications[22-24]. In the bisulfite conversion reaction, DNA is first treated with sodium bisulfite to convert cytosine residues into uracil in denatured (single-stranded) DNA, under conditions whereby 5-mC and 5-hmC remain essentially non-reactive. The DNA is then amplified by PCR where the uracils are converted to thymines. Bisulfite converted DNA can be analyzed for gene- or allele-specific methylation patterns, or adapted for genome-wide DNA methylation analysis. A methylation profile of the sample can then be created by comparing the sequence of the converted DNA to untreated DNA.

Active Motif's Bisulfite Conversion Kit simplifies analysis of DNA methylation by providing optimized conversion reagents and an easy-to-use protocol. The conversion reaction can be performed in as little as 1.5 hours with a 99% conversion efficiency of unmethylated cytosines to uracils. The Bisulfite Conversion Kit includes a positive control conversion-specific PCR primer pair that is specific for bisulfite-converted human and mouse DNA. Because the primer pair produces a PCR product only if conversion has occurred, you can validate the success of the conversion reaction before spending extra time and money on sequencing.

Traditional Methods to Study DNA Methylation

To date, there are several methods used for methylation analysis:

1. **Methylation-sensitive restriction enzyme analysis:** Isoschizomers of bacterial restriction endonucleases with different sensitivities for methylated DNA can be used to determine the methylation status of specific CpG-dinucleotides[25]. Pairs of isoschizomers are used that recognize the same restriction site, but one enzyme is unable to cleave methylated DNA. Methylation-sensitive restriction enzymes have several limitations including that methylation-sensitive restriction merely informs on the methylation status of the cytosine residues which are recognized by the restriction enzymes used.
2. **Bisulfite conversion:** Bisulfite conversion[19-21] consists of the treatment of genomic DNA with sodium bisulfite, leading to deamination of unmethylated cytosines into uracil. PCR is then performed with primers that differentiate between methylated and unmethylated sequences. Bisulfite converted DNA can be sequenced in order to quantify the level of DNA methylation in the sample. However, bisulfite-based techniques do involve chemical treatments that can damage DNA and limit throughput. Additionally, PCR primer design becomes difficult due to reduction in genome complexity after bisulfite treatment.
3. **Methylated DNA Immunoprecipitation (MeDIP):** In this assay, an antibody specific for methylated cytosines (anti-5-methylcytosine antibody or anti-5-hydroxymethylcytosine antibody) is used to immunoprecipitate methylated DNA from genomic DNA fragmented by enzymatic digestion or sonication[26]. The resulting enrichment is usually analyzed by PCR based methods; thus MeDIP can be combined with DNA microarrays or adapted with sequencing primers for genome-wide analysis of CpG methylation. However, this technique is relatively time-consuming and requires a large amount of fragmented DNA starting material and does not give single-base-pair resolution.
4. **Methyl-CpG Binding proteins:** This family of proteins takes its definition from the methyl-CpG binding domain (MBD), the minimum portion with specific affinity for a single, symmetrically methylated CpG pair. The MBD2b protein has been found to possess one of the highest affinities for methylated DNA among MBD proteins and has the greatest capacity to differentiate between methylated and unmethylated DNA[27]. The Methylated CpG Island Recovery Assay (MIRA) utilizes the combination of MBD2b with its binding partner MBD3L1, methyl-CpG-binding protein 3-like-1, to generate a higher affinity for methylated DNA than MBD2b protein alone[28,29]. Methyl-CpG binding proteins are limited to the evaluation of methylated DNA in a CpG context as the proteins do not recognize methylated cytosines that exist outside of a CpG dinucleotide. The MBD2 proteins are only capable of binding to 5-mC methylation; they cannot be used to enrich for 5-hmC methylation[24].

References

1. Ramsahoye, B.H. *et al.* (2000) *Proc. Natl Acad. Sci. USA*, 97: 5237-5242.
2. Lister, R. *et al.* (2009) N*ature*, 462: 315-322.
3. Antequera, F. and Bird, A. (1993) *Proc. Natl Acad. Sci. USA*, 90: 11995-11999.
4. Bird, A. (2002) *Genes Dev*, 16: 6-21.
5. Issa, J.P. (2004) *Nat Rev Cancer*, 4: 988-993.
6. Toyota M., *et al.* (1999) *Proc. Natl Acad. Sci. USA*, 96: 8681-8686.
7. Hughes, L.A. *et al.* (2012) *Biochim Biophys Acta.*, 1825: 77-85.
8. Samowitz, W.S. (2007) *Mol Diagn*., 9: 281-283.
9. Marsit, C.J. *et al.* (2006) *Cancer Res*., 66: 10621-10629.
10. Suzuki, M. *et al.* (2006) *Cancer*, 106: 2200-2207.
11. Shen, L. *et al.* (2002) *J Natl Cancer Inst*., 94: 755-761.
12. Fang, F. *et al.* (2011) *Sci Transl Med*., 3:75ra25.
13. Jones, P.A. and Baylin, S.B. (2002) *Nature Rev. Genet.*, 3: 415-428.
14. Esteller, M. *et al.* (2001) *Cancer Res.*, 61: 3225-3229.
15. Laird, P.W. (2003) *Nat Rev Cancer,* 3: 253-266.
16. Van Engeland, M. *et al.* (2011) *J Clin Oncol*., 29: 1382-1391.
17. Esteller, M. (2008) *N Engl J Med*., 358: 1148-1159.
18. Reik, W. *et al.* (2001) *Science*, 293: 1089-1093.
19. Shapiro, R. *et al.* (1974) *J Am Chem Soc*, 96(3): 206-212.
20. Frommer, M. *et al.* (1992) *Proc. Natl Acad. Sci. USA*, 89: 1827-1831.
21. Clark, S.J. *et al.* (1994) *Nuc. Acids Res.,* 22: 2990-2997.
22. Huang, Y. *et al.* (2010) *PLoS One*, 26: e8888.
23. Jun, S. *et al.* (2010) *Nucl. Acids Res*., 38: e125.
24. Nestor, C. *et al.* (2010) *BioTechniques*, 48: 317-319.
25. Dalh, C. and Guldberg, P. (2003) *Biogerontology*, 4: 233-250.
26. Weber, M. *et al.* (2005) *Nat. Genet*., 37: 853-832.
27. Fraga, M.F. *et al.* (2003) *Nucleic Acids Res*., 31: 1765-1774.
28. Rauch, T. and Pfeifer, G. (2005) *Lab. Investigation*, 85: 1172-1180.
29. Jiang, C.L. *et al.* (2004) *J. Bio. Chem*., 279: 52456-52464.

Kit Performance and Benefits

The Bisulfite Conversion Kit is for research use only. Not for use in diagnostic procedures.

Sensitivity: Bisulfite Conversion can be performed on 200 pg - 2 µg of DNA, with 500 ng to 2 µg optimal.

Conversion efficiency: >99%

DNA Recovery: >80%

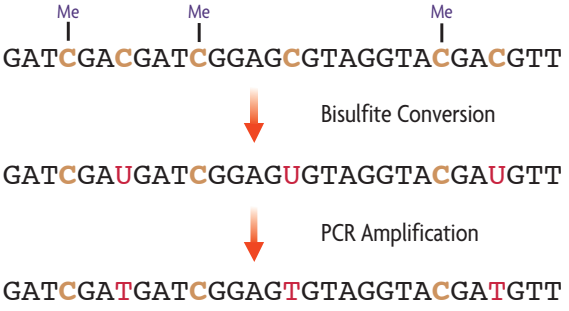


Figure 1: DNA sequence following bisulfite conversion and PCR amplification.

This figure shows an example of the effect of bisulfite treatment on a DNA sequence. The original DNA sequence contains 6 cytosine residues, 3 methylated (Me) and 3 unmethylated. Following bisulfite conversion the unmethylated cytosines are converted into uracil while the methylated cytosines remain as cytosine. The methylation profile of the DNA can then be determined by PCR amplification followed by DNA sequencing, where the uracils will be detected as thymine. This is why it is often stated that bisulfite treatment converts C to T.

Kit Components and Storage

Bisulfite Conversion Kit components arrive at room temperature and can be stored at 4°C prior to first use. Then, we recommend storing each component at the temperatures listed in the table below. Components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
Conversion Reagent	5 tubes	RT
Denaturation Reagent	875 µl	RT
Hydroquinone	5 x 100 µl	RT
Buffer A	1.75 ml	RT
Buffer B	1.1 ml	RT
DNA Binding Buffer	2 x 15 ml	RT
DNA Wash Buffer	25 ml	RT
DNA Elution Buffer	2.5 ml	RT
DNA purification columns	50 ea	RT
DNA column collection tubes	50 ea	RT
Proteinase K	2 mg	-20°C
Conversion-specific PCR primer pair (4 µM)	200 µl	-20°C

Additional Materials Required

Bisulfite Conversion and PCR Analysis

- Purified sample DNA
- 0.2 ml PCR tubes and caps
- 100% isopropanol
- Microcentrifuge tubes and microcentrifuge
- dNTP mixture (10 mM each)
- Thermocycler
- Hot Start *Taq* polymerase (5 U/µl) and PCR reaction buffer recommended for use with bisulfite converted DNA (*e.g.* Qiagen HotStarTaq DNA polymerase, Catalog No. 203203)
- Methylation-specific PCR primers for the gene of interest
- 2% agarose TAE gel
- Gel apparatus and power supply
- 6x Loading dye
- Molecular weight marker

DNA Clean Up

- (Optional) DNA purification kit (*e.g.* QIAquick PCR purification kit, Qiagen Cat. No. 28104)
- Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v)
- 3 M Sodium acetate, pH 5.5
- Glycogen, molecular biology grade (20 ng/µl) or tRNA (10 µg/µl)
- 100% ethanol

DNA Cloning and Sequencing

- Ligation vector (*e.g.* Topo TA Cloning Kit for sequencing, Life Technologies Cat. No. 450030)
- Competent *E.coli* cells (*e.g.* RapidTrans™ TAM 1, Active Motif Cat. No. 11096)
- Bacterial growth media and plates with appropriate selection
- DNA miniprep materials
- DNA sequencing reagents

Buffer Preparation

Input DNA

Input DNA should be in the range of 200 pg to 2 µg, with the optimal range being 500 ng to 2 µg. Please see the Appendix A: Troubleshooting section for suggestions on adding a carrier when using less than 100 ng of DNA.

Preparation of Proteinase K working solution

To improve the efficiency of the bisulfite conversion reaction the Input DNA is treated with Proteinase K prior to performing the conversion reaction. Proteinase K is supplied lyophilized and can be stored at 4°C prior to first use. Resuspend 2 mg of Proteinase K in 100 µl sterile water. Vortex to fully resuspend. The final concentration of the Proteinase K working solution is 20 mg/ml and should be stored at -20°C.

Preparation of Conversion Buffer

Five tubes of Conversion Reagent are provided in the Bisulfite Conversion Kit. Each vial is sufficient for performing 10 bisulfite conversions. Prepare the Conversion Buffer by resuspending one of the Conversion Reagent tubes with 700 µl dH_2O, 350 µl Buffer A and 175 µl Denaturation Reagent. Mix at room temperature for 10 minutes with intermittent vortexing.

NOTE: This is a saturated solution and it is normal for the Conversion Reagent to not dissolve completely. The Conversion Buffer is stable for 1 week at -20°C. Conversion Reagent is sensitive to air and moisture. Keep cap on tight and re-cap immediately after use.

Preparation of Hydroquinone

Hydroquinone is an alkalizing agent and prevents DNA from strand breakage because of depurination. Each tube provided can be used for 10 conversion reactions. Prepare a working stock of Hydroquinone by resuspending one tube with 100 µl dH_2O. Keep mixture in dark and discard after use.

Preparation of Desulfonation Buffer

For each reaction, combine 22 µl Buffer B with 88 µl dH_2O and 110 µl 100% isopropanol. If this reagent will not be used immediately, it can be stored for up to one week at -20°C.

DNA Binding Buffer

Is supplied ready to use.

DNA Wash Buffer

Is supplied ready to use.

DNA Elution Buffer

Is supplied ready to use.

Positive control Conversion-specific PCR primer pair

The Bisulfite Conversion Kit's positive control Conversion-specific PCR primer pair (4 µM) is specific for amplification of bisulfite converted human or mouse DNA. This primer pair should amplify DNA that has been successfully bisulfite converted to generate a 220 bp PCR product. It should not generate a PCR product with untreated human or mouse DNA. If using a different species of DNA besides human or mouse, the included positive control Conversion-specific PCR primer pair will not work and conversion-specific primers will need to be designed to your species of interest.

Design of PCR primers

PCR primer design is critical for successful analysis of converted DNA. There are several sites that offer information on designing methylation-specific PCR primers. The MethPrimer website: http:// www.urogene.org/methprimer/ is a free site that contains several useful tools for the design of bisulfite-conversion-based Methylation PCR Primers. Additional programs include, MethylViewer http://dna.leeds.ac.uk/methylviewer/ and PREMIER Biosoft International offers the Beacon Designer program http://www.premierbiosoft.com/crm/jsp/com/pbi/crm/ clientside/ ProductList.jsp. We suggest designing both bisulfite conversion specific and untreated PCR primer pairs for each gene of interest to validate the success of the conversion reaction prior to downstream analysis. For design of primers to be used in the same PCR reaction as the positive control Conversion-Specific PCR primer pair we recommend an annealing temperature between 56-60°C.

Bisulfite Conversion Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

The Bisulfite Conversion Kit can efficiently convert DNA samples containing between 200 pg to 2 µg of DNA, with the optimal input range of 500 ng to 2 µg. The positive control Conversion-specific PCR primer pair is designed to anneal to bisulfite converted human and mouse DNA. If you plan to perform the positive control reactions, it is recommended that you perform the conversion reaction using 500 ng of human or mouse DNA.

Step A: Proteinase K Treatment

1. Add sample DNA to a microcentrifuge tube for proteinase K treatment. Treatment with proteinase K can be performed in large scale using the entire mass of DNA recovered from sample extraction. Alternatively, for smaller scale treatment, add the appropriate amount of DNA that will be used for bisulfite conversion to the microcentrifuge tube.
2. Add Proteinase K working solution (20 mg/ml) to the DNA to achieve a final concentration of 50 µg/ml. (For example if using 1 ml DNA, add 2.5 µl Proteinase K working solution.) If the volumes needed are too small to accurately pipet, prepare an additional 1:10 dilution of the Proteinase K working solution in sterile water just before use to make a 2 mg/ml stock.
3. Incubate the DNA with Proteinase K at 50°C for a minimum of 30 minutes. Longer incubation times, including overnight, may also be performed at 50°C.

Step B: Conversion Reaction

1. Prepare Conversion Buffer and Hydroquinone as described in the Buffer Preparation Section on page 9.
2. Set up a conversion reaction for each sample by adding the reagents in the order listed below into PCR tubes. Mix well by pipetting. The final volume should be 140 µl. If using less than 13 µl of DNA, adjust the volume with water to a final volume of 140 µl. Keep hydroquinone away from light.

Reagent	Quantity
DNA	13 µl
dH2O	X µl
Hydroquinone	7 µl
Conversion Buffer	120 µl
Total Volume	**140 µl**

3. Place tubes in the thermocycler and set the following program. To prevent evaporation of samples, use the heated lid or overlay reactions with mineral oil.

   95°C for 30 seconds
   58°C for 20 minutes
   (95°C for 10 seconds, 58°C for 20 minutes) for 3 cycles
   Hold at 4°C

   Note: The suggested 1.5 hour conversion PCR conditions are ideal for undigested genomic DNA. The number of cycles can be increased if incomplete conversion is a concern, however, incubation periods longer than 3 hours are not recommended. If using fragmented DNA, or large quantities of genomic DNA, please refer to Appendix Section A: Troubleshooting Guide on page 18 for alternative bisulfite conversion conditions.

4. Remove tubes and continue with Step C below. The protocol can also be stopped here and DNA samples can be kept at 4°C in the dark for up to 5 days.

Step C: On-column Desulfonation and DNA Purification

1. Prepare Desulfonation Buffer as described in the Buffer Preparation section on page 9.
2. For each conversion reaction, aliquot 500 µl DNA Binding Buffer into a 1.5 ml microcentrifuge tube. Add the entire conversion reaction mixture to the DNA Binding Buffer in the tube and vortex.
3. Remove the desired number of DNA purification columns and place each one in a collection tube.
4. Pipet each DNA Binding Buffer/conversion reaction mixture into one of the DNA Purification columns and spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
5. Add 200 µl DNA Wash Buffer to each column and spin at 10,000 rpm for 30 seconds in a microcentrifuge.
6. Add 200 µl Desulfonation Buffer to each column and incubate 20 minutes at room temperature. Spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
7. Add 200 µl DNA Wash Buffer to each column and spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
8. To remove any residual wash buffer, spin at 10,000 rpm for 30 seconds in a microcentrifuge. Remove the column and place in a new 1.5 ml microcentrifuge tube.
9. Add 30 µl DNA Elution Buffer directly to the filter of the column.
10. Incubate 3 minutes at room temperature. Spin at 10,000 rpm for 30 seconds in a microcentrifuge. The eluate will contain the converted DNA.
11. The eluted DNA is now ready for downstream analysis.

Optional Downstream Analysis

Step D: Quantification of Bisulfite Converted DNA

Following bisulfite treatment of DNA, the original base-pairing of DNA no longer exists since the unmethylated cytosine residues are converted into uracil. If using a spectrophotometer, set the unit to measure RNA absorption (using a conversion of 40 µg/ml for A260 nm = 1.0) because bisulfite converted DNA resembles RNA since it contains uracil and is largely single stranded. Alternatively, use a Qubit ssDNA Assay or check the quality of the bisulfite converted DNA using Agilent Bioanalyzer with RNA 6000 Pico Kit or similar detection methods. However, if the bisulfite DNA is PCR amplified prior to quantification, traditional methods to quantify DNA can be used.

Step E: Conversion-Specific PCR

Determine the number of conversion-specific PCR reactions to perform. The included positive control Conversion-specific PCR primer pair can be used to quickly assess the success of the bisulfite conversion reaction using human or mouse DNA samples. For conversion-specific gene analysis, use a conversion-specific PCR primer pair and an untreated PCR primer pair (if desired) for each gene locus of interest. Follow the recommendation listed on page 10 to design bisulfite conversion-specific primers for your gene of interest. We recommend an annealing temperature between 56-60°C if the primers are to be used in the same PCR reaction as the positive control Conversion-Specific PCR primer pair.

We also recommend performing a Hot Start PCR reaction when working with bisulfite converted DNA to ensure reliable and consistent amplification of the DNA template.

DNA Template	Positive Control Conversion-specific Primer Pair	Gene-specific Converted Primer Pair	Gene-specific Untreated Primer Pair
Bisulfite Converted DNA	Rxn 1	Rxn 4	Rxn 7
Untreated DNA	Rxn 2	Rxn 5	Rxn 8
H_2O control	Rxn 3	Rxn 6	Rxn 9

1. Label the desired number of PCR tubes and place in a PCR tube rack on ice.
2. Make a 20 ng/µl dilution of unconverted genomic DNA (the same Input DNA that was used for the conversion reaction).
3. Prepare a PCR master mix for each of the primer sets to be tested in 1.5 ml microcentrifuge tubes as described below. DNA templates will be added later directly to the PCR tubes. Combine components on ice and mix by vortexing. Four reactions are prepared to account for any loss during pipetting. The table below is provided as an example, please adjust as

needed to follow the recommendation of the Hot Start PCR polymerase to be used.

Reagent	One Rxn	Master Mix
dH_2O	13.55 µl	54.2 µl
PCR primer pair (4 µM)	2 µl	8 µl
dNTP mixture (10 mM each dNTP)	0.25 µl	1 µl
10X PCR Buffer	2 µl	8 µl
Hot Start *Taq* (5 U/µl)	0.2 µl	0.8 µl
Total Volume (Not including DNA template)	18 µl	72 µl

4. Aliquot 18 µl of this PCR cocktail into the appropriately labeled tubes on ice.
5. Add template DNA or water to the PCR tubes as indicated below and mix. Ensure that the entire 20 µl PCR reaction is on the bottom of the tube. Centrifuge tubes if necessary.

PCR Rxn #	PCR Master Mix	DNA Template	dH_2O
1, 4 & 7	18 µl	2 µl Bisulfite Converted DNA	–
2, 5 & 8	18 µl	2 µl Untreated DNA (20 ng/µl)	–
3, 6 & 9	18 µl	–	2 µl

6. Place tubes in PCR cycler and program the thermocycler as below. (To prevent evaporation of samples, use the heated lid or overlay reactions with mineral oil.)

   94°C for 15 minutes
   (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute) for 30–35 cycles
   72°C for 10 minutes
   Hold at 4°C

Step F: Agarose Gel Analysis of PCR Products

1. Pour a 2% agarose gel in 1X TAE.
2. Add 5 µl from each of the PCR reactions to 1 µl 6X DNA loading dye and load sample on a gel. Use a 100 or 50 bp DNA ladder. Run the gel until the marker is near the bottom of the gel and the markers are well separated. Only the PCR reactions containing bisulfite converted DNA should show a PCR product when using conversion-specific primer sets. Untreated DNA and the H_2O control PCR reactions should not have any PCR amplification. For the positive control Conversion-specific PCR primer pair, the bisulfite converted DNA should generate a 220 bp amplicon as shown in Figure 2 on the next page.

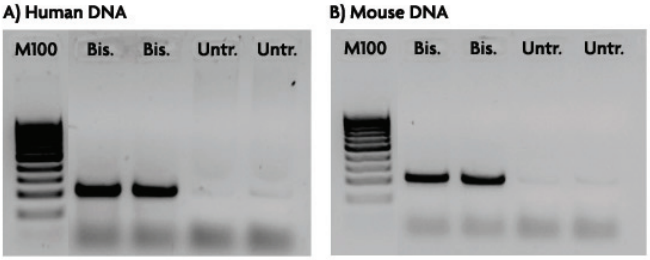


Figure 2: Positive Control Conversion-specific PCR primer pair is specific for converted human or mouse DNA.
The figure above depicts the PCR results of human (A) and mouse (B) genomic DNA that was either bisulfite converted (Bis.) according to the assay protocol or untreated (Untr.) and amplified using the included positive control Conversion-specific PCR primer pair. The primer pair is specific towards bisulfite converted DNA and therefore a 220 bp PCR amplicon is present in the bisulfite treated DNA samples and not in the untreated DNA samples.

Step G: DNA Clean Up

Prior to cloning it is necessary to clean up the DNA. Use the following protocol to perform a phenol/chloroform extraction followed by ethanol precipitation. Alternatively, DNA can be purified using columns such as QIAquick PCR purification kit (Qiagen part no. 28104). Elute in 30-50 µl volume. If more than one band is amplified by the PCR reaction in Step E, the DNA band of the expected size should be gel-purified using a gel extraction kit such as QIAEX II gel extraction kit (Qiagen part no. 20021).

1. To the remaining PCR reaction from Step E, add the necessary amount of sterile water to make the total volume 100 µl.
2. Add an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v) to the sample.
3. Vortex the tube at maximum speed for 15 seconds.
4. Centrifuge the tube for 5 minutes at 12,000 x *g* at room temperature.
5. Carefully transfer the top aqueous phase to clean microcentrifuge tube without collecting any of the lower organic phase or precipitate that may occur between the phases.
6. To each sample add:

   1 µl Glycogen (20 ng/µl)

   1/10 sample volume of 3 M sodium acetate pH 5.5

   2.5 sample volumes of 100% ethanol
7. Mix well and incubate at -80°C for at least 2 hours.
8. Centrifuge the tube for 20 minutes at 12,000 x *g*, 4°C.
9. Carefully discard the supernatant without disturbing the pellet.
10. Add 500 µl of cold 70% ethanol.

11. Centrifuge the tube for 10 minutes at 12,000 x *g*, 4°C.
12. Carefully discard the supernatant without disturbing the pellet.
13. Air-dry the pellet for 5-10 minutes (do not completely dry the pellet).
14. Resuspend the DNA pellet in 30 µl sterile DNase-free water.
15. This eluted DNA can be used immediately in cloning or stored at -20°C. If the DNA is stored at -20°C, it will need to be reheated at 37°C for 10 minutes prior to use to ensure DNA does not stick to the walls of the tube.

Step H: DNA Cloning

1. The cleaned PCR product can be cloned into a DNA vector of choice according to the instructions of the manufacturer (*e.g.* Topo TA Cloning Kit for sequencing, Life Technologies Cat. No. 450030). If possible, plasmids containing the *Lac*Z gene for blue/white screening are recommended. An example ligation reaction is shown below, but please follow the recommendations associated with the DNA vector of choice.

Reagent	Quantity
DNA template	4 µl
Ligation Buffer	1 µl
DNA vector	1 µl
Total Volume	**6 µl**

Incubate 5 minutes at room temperature.

2. Transform competent bacterial cells, such as RapidTrans™ TAM 1 (Active Motif Cat. No. 11096), with 2 µl of the ligation reaction. Follow the appropriate protocol for the competent cells used. The RapidTrans™ TAM 1 chemically competent cell protocol is listed below as an example.
    a. Remove required number of transformation reaction tubes from –80°C storage and place on ice to thaw.
    b. Add 1-5 µl of plasmid DNA/ligation to thawed cells. Mix by tapping tubes gently and replace on ice immediately. Do not mix by vortexing or pipetting. Do not add more than 5 µl (10% of competent cell volume) to reaction.
    c. Incubate transformation reactions on ice for 30 minutes.
    d. Heat-shock the tubes by immersing in a 42°C water bath for exactly 30 seconds.
    e. Replace transformation reactions on ice for 2 minutes.
    f. Aseptically add 250 µl SOC medium to each reaction.
    g. Incubate tubes at 37°C for 1 hour with shaking at 225-250 rpm.

h. Using a sterile spreader, plate out 20-200 µl of each transformation on pre-warmed LB agar plates. If using blue/white screening, ensure that the selective plate contains X-Gal. For preparation of X-gal Stock solution and plates see note below.

Note: Make a 40 mg/ml solution of X-gal in dimethylformamide (DMF). Use a glass or polypropylene tube and wrap in aluminum foil to prevent damage by light. Store at –20°C. Plate 40 µl of X-gal stock solution on top of the LB agar and let stand for approximately 1 hour. Alternatively, add the X-gal to the molten LB agar prior to pouring plates.

i. Allow plates to completely absorb any excess media.

j. Incubate inverted plates overnight at 37°C.

3. Select 10 colonies for screening. Select white colonies if using blue/white screening as the expression of *Lac*Z has been interrupted with the insertion of the ligation product.
4. Isolate plasmid DNA of the 10 selected colonies using any commercially available kit or published protocol.
5. Analyze the isolated DNA using restriction enzyme analysis to confirm the presence of the correct insert, or by PCR using region-specific primers or primers corresponding to sequences within the vector (*e.g.* M13 forward and reverse primers)
6. Submit DNA from the positive clones for sequencing. Use forward and reverse primers based on the DNA vector used for cloning. It is recommended to submit 10 colonies for sequencing to ensure representative results. It is also necessary to submit unconverted DNA for sequencing comparison.

Step I: Analyzing the Sequencing Results

1. If the CpG dinucleotide was methylated in the original DNA, the sequencing read will show a CG at that location. If the CpG dinucleotide was unmethylated in the original DNA, the bisulfite conversion will have converted the cytosine to uracil and the sequencing read will show a TG at that location.
2. Compare the bisulfite converted sequence data against the unconverted DNA sequence.
3. Quantify the number of methylation sites for the region of interest to determine the percentage of methylation.

Appendix

Section J. Troubleshooting Guide

Problem/question	Recommendation
Using less than 100 ng of DNA as starting material	When converting a small amount of DNA, add 1 µg Glycogen (20 ng/µl) or tRNA (10 µg/µl) as a carrier to the 500 µl DNA Binding Buffer for each reaction during the on-column desulfonation and DNA purification step to minimize sample loss.
Starting DNA volume is greater than 13 µl	DNA volumes up to 20 µl can be used without affecting the protocol
Conversion time	Large quantities of DNA may require longer conversion time to achieve complete conversion. For quantities of DNA greater than 1 µg, or if using DNA that is fragmented, we recommend performing a 5 hour incubation at 50°C to efficiently convert DNA samples. We do not recommend using less than 500 ng of input DNA if the sample material is fragmented as this will lead to reduced DNA recovery. For DNA sequences that are GC-rich and/or contain extensive secondary structures we suggest a 9 hour conversion at 50°C. Longer conversion times, up to 16 hours, may be used if noticing incomplete conversion, however, this may result in degradation of the DNA. If noticing a loss of DNA after the conversion reaction, the conversion time can be reduced to 3 hours at 50°C.
No PCR product	Conversion reagent is sensitive to air and moisture. If conversion reagent was not properly sealed and stored, repeat conversion reaction using a new tube of conversion reagent.
	Test primers on control bisulfite converted DNA. Evaluate the optimal PCR conditions including annealing temperature, primer and magnesium concentrations. Consider designing new primers.
	We recommend performing a Hot Start PCR reaction using a polymerase that has been validated to work with bisulfite converted DNA. Follow the recommendations of the manufacturer for the preparation of the PCR reaction.
	If working with low amounts of Input DNA, such as 200 pg, it may be difficult to PCR amplify the bisulfite converted DNA for visualization on an agarose gel. Concentrate DNA prior to PCR to use more DNA per reaction, or use a different analysis method.
Can I store my conversion reaction samples for longer than 5 days before performing the DNA purification and desulfonation steps?	The DNA is recommended to be stored at 4°C for 5 days. If longer storage is needed, store samples at -20°C. As precipitates can form during -20°C, long term storage is not recommended.
Storage of converted DNA	Once DNA is prepared using Bisulfite Conversion Kit, samples may be stored at -20°C prior to PCR analysis
How do I quantify my bisulfite converted DNA?	Bisulfite converted DNA will resemble RNA as it is single stranded and contains uracil in place of the unmethylated cytosines. Use a spectrophotometer set to measure RNA with a conversion factor of 40 µg/ml for A260nm = 1.0. However, if the bisulfite converted DNA has been PCR amplified prior to quantification, traditional methods to quantify DNA can be used.

Section K. Related Products

DNA Methylation	Format	Catalog No.
Bisulfite Conversion Kit	50 rxns	55016
MethylCollector™ Ultra	30 rxns	55005
HypoMethylCollector™	30 rxns	55004
MeDIP	10 rxns	55009
Hydroxymethyl Collector™	25 rxns	55013
hMeDIP	10 rxns	55010
PvuRts1I restriction enzyme	50 units	55011
β-Glucosyltransferase enzyme	500 units	55012
Recombinant TET1 protein, active	25 µg	31363
DNMT Activity / Inhibition Assay	1 x 96 rxns	55006
Methylated DNA Standard Kit	3 x 2.5 µg	55008
5-Carboxylcytosine DNA Standard	0.5 µg	55014
Fully Methylated Jurkat DNA	10 µg	55003
Jurkat genomic DNA	10 µg	55007

Antibodies	Application	Format	Catalog No.
3-Methylcytosine rabbit pAb	DB	100 µg	61111
5-Carboxylcytosine rabbit pAb	DB, IF	100 µl	61225
5-Formylcytosine rabbit pAb	DB, IF	100 µl	61223
5-Hydroxymethylcytidine mouse mAb	DB, MeDIP	100 µg	39999
5-Hydroxymethylcytidine rabbit pAb	DB, IF, IHC, MeDIP	100 µl	39769
5-Methylcytosine mouse mAb	DB, FACS, IHC, IP, MeDIP	50 µg	39649
5-Methylcytosine rabbit pAb	DB, IP, MeDIP	100 µg	61255
CGBP rabbit pAb	WB	200 µl	39203
DNMT1 mouse mAb	ChIP, IHC, IP, WB	100 µg	39204
DNMT2 rabbit pAb	WB	100 µg	39205
DNMT3A mouse mAb	ChIP, IF, IHC, WB	100 µg	39206
DNMT3B mouse mAb	ChIP, IF, IP, WB	100 µg	39207
DNMT3L rabbit pAb	WB	100 µl	39907
Kaiso mouse mAb	WB	200 µg	39365
MBD1 mouse mAb	WB	100 µg	39215
MBD2 rabbit pAb	WB	200 µl	39547
MBD3 mouse mAb	WB	100 µg	39216
MBD4 rabbit pAb	WB	100 µg	39217
MeCP2 rabbit pAb	WB	100 µg	39218
MeCP2 mouse mAb	ChIP, IF, IHC, IP, WB	100 µg	61291
Tet1 rabbit pAb	ChIP, WB	100 µl	61443
Tet2 mouse mAb	IP, WB	100 µg	61389
Tet3 rabbit pAb	WB	100 µl	61395
Ubiquitin mouse mAb	IF, IHC, IP, WB	100 µg	39741
Uhrf1 rabbit pAb	WB	200 µl	39625

For more information or to see a complete list of DNA methylation antibodies, please visit our website at **www.activemotif.com/methylabs**.

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

Active Motif North America
1914 Palomar Oaks Way, Suite 150
Carlsbad, CA 92008
USA
Toll Free: 877 222 9543
Telephone: 760 431 1263
Fax: 760 431 1351
E-mail: tech_service@activemotif.com

Active Motif Europe
Avenue Reine Astrid, 92
B-1310 La Hulpe, Belgium
UK Free Phone: 0800 169 31 47
France Free Phone: 0800 90 99 79
Germany Free Phone: 0800 181 99 10
Telephone: +32 (0)2 653 0001
Fax: +32 (0)2 653 0050
E-mail: eurotech@activemotif.com

Active Motif Japan
Azuma Bldg, 7th Floor
2-21 Ageba-Cho, Shinjuku-Ku
Tokyo, 162-0824, Japan
Telephone: +81 3 5225 3638
Fax: +81 3 5261 8733
E-mail: japantech@activemotif.com

Visit Active Motif on the worldwide web at **http://www.activemotif.com**

At this site:

- Read about who we are, where we are, and what we do
- Review data supporting our products and the latest updates
- Enter your name into our mailing list to receive our catalog, *MotifVations* newsletter and notification of our upcoming products
- Share your ideas and results with us
- View our job opportunities

Don't forget to bookmark our site for easy reference!