methylSEQr™ System: Bisulfite Conversion and Sequencing of Methylated DNA

DNA methylation patterns—are often considered as a second code, an additional layer of information superimposed on the DNA code that determines many phenotypic attributes. The DNA code is stable, but DNA methylation patterns change in response to spatial, temporal and environmental cues.

After PCR amplification, the sequence of the bisulfite converted DNA will have C residues only if the C was methylated and will occur adjacent to G's (CpG). All other non-methylated C's will be detected as T's. An unmethylated gDNA sample will have no C's in the sequencing data.

Comparison of sodium bisulfite treated DNA sequences with sequences obtained from untreated genomic DNA allows the precise identification of all methylated cytosines within a stretch of DNA.

Protocol Summary

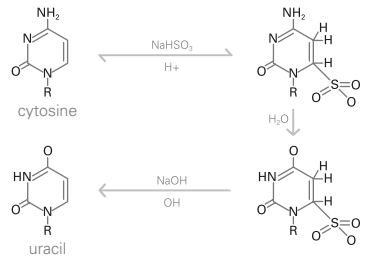
The protocol for methylation sequencing is divided into three main stages:

- Sample Prep and bisulfite conversion
- PCR template Prep
- Sequencing.

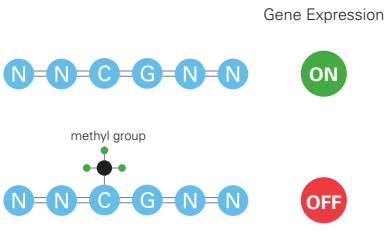
PCR amplicons generated after bisulfite conversion can be cloned and sequenced or sequenced directly.

The protocol, as seen on the following page, is based on the methylSEQr™ Bisulfite Conversion Kit. For more details please refer to the Protocol for the kit.

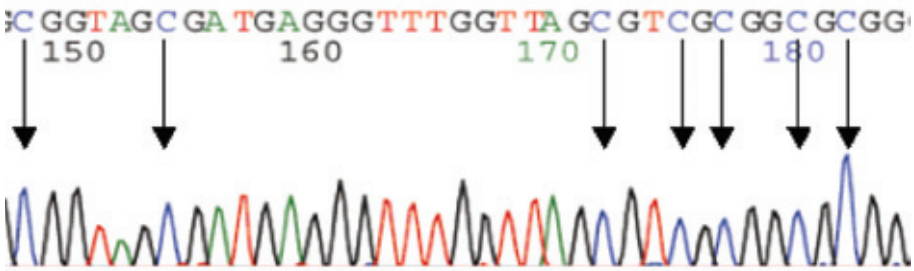
The methylSEQr™ Bisulfite Conversion Kit uses a straightforward procedure based on conveniently packaged bisulfite-conversion reagents and an efficient purification scheme which reliably provides a high recovery of the bisulfite-converted gDNA. Bisulfite treatment converts non-methylated cytosine (C) to uracil (U). Methylated C residues are protected from the conversion. Non-methylated C residues are detected by the C to T transition in the treated DNA sequence. DNA methylation is a common biochemical modification of eukaryotic DNA. Selective gene inactivation has been shown to be due to the DNA methylation of cytosines in the promoter regions.



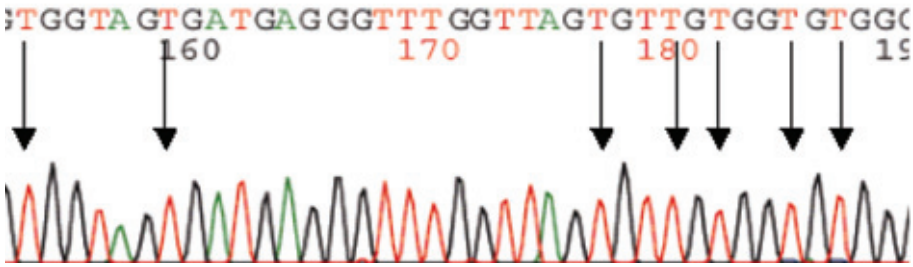
Sodium bisulfite deaminates cytosine to produce Uracil. Methylated cytosines are protected from this conversion.



The methylation-susceptible cytosines occur next to Guanine residues as a CpG di-nucleotide. CpG islands (CpG island = clusters of CpG's with a GC content of > 55% in < 500 bp regions) are often found in the regulatory regions of genes.



DNA sequence from a fully methylated DNA after methylSEQr™ Kit conversion (methyl C residues are retained as C after bisulfite conversion, PCR, and sequencing).



DNA sequence from fully non-methylated DNA after methylSEQr™ Kit conversion. Arrows show that all the Cs in the unmethylated sample are converted to Ts.

methylSEQr™ Kit Protocol:

1. Genomic DNA

a) Prepare genomic DNA according to standard protocols.

b) Determine the concentration by A260 or fluorescence.

2. Bisulfite Conversion

a) To <300 ng genomic DNA in 45 µl H_2O, add 5 µl of MethylSEQr™ Denaturation Buffer. Mix well. Incubate at 37°C for >15 min.

b) Add 100 µl of freshly reconstituted MethylSEQr™ Conversion Reagent to the denatured genomic DNA sample, mix. Incubate in the dark at 50°C for 12–16 hrs.

3. Cleanup

a) Centrifuge the DNA + Conversion reagent mixture through the methylSEQr™ column (500xG/15 min).

b) Wash with two changes of 350 µL water. Discard filtrate.

c) Add 350 µL of 0.1M NaOH. Incubate 5 min. Centrifuge (500xG/15 min). Discard the filtrate.

e) Wash with 350 µL water. Discard filtrate.

f) Add 50 µL 0.1 x TE (10 mM Tris/HCl pH 8, 0.1 mM EDTA) to the upper chamber and mix by pipetting up and down. Incubate 5 min. Invert column and collect bisulfite treated gDNA by centrifuging at 1000xG for 30 sec.

4. Template Prep

a) Primer Design: Applied Biosystems offers a free design tool, Methyl Primer Express® Software v1.0 for the design of PCR primers for bisulfite sequencing and for methylation-specific PCR (MSP). Methyl Primer Express® software can be downloaded from **www.appliedbiosystems.com/methylprimerexpress**

b) PCR reaction: We recommend using primers that have been tailed with –21 M13 forward and reverse primer sequences. Prepare the following reaction mix: AmpliTaq Gold® 10 x buffer, 1 µL; dNTP 2.5 mM each, 0.8 µL, MgCl2 25 mM 0.8 µL, AmpliTaq Gold® DNA Polymerase 0.2 µL, Forward primer 5 µM, 0.25 µL, Rev. primer 5 µM, 0.25 µL, methylSEQr™ system treated DNA (10ng/µL) 0.5 µL, water to 10 µL.

Suggested thermocycling conditions with the AB 9700: Heat activation, 95ºC for 5 min, followed by 40 cycles of 97ºC for 5 sec, 50-60ºC annealing (dependent on primer T_m) for 120 sec, and 72ºC extension for 45 sec. Followed by a final extension of 72ºC for 10 min. Hold at 4ºC. A two-stage PCR with the first 5 cycles at the recommended annealing temperature and the remaining 35 cycles at a 10ºC higher annealing temperature may increase the efficiency of the PCR.

c) PCR cleanup: Add 2 µL of ExoSAP-IT® Reagent (USB Corporation), incubate at 37ºC for 30 min followed by heat inactivation at 80ºC for 15 min.

5. Sequencing

a) Sequencing Reaction: For 10 µl sequencing reactions – BigDye® Terminator Ready Reaction Mix v3.1, 4.0 µL; M13 forward or reverse primer (3.2 pmol/µL), 1.0 µL; Deionized water, 3.0 µL; PCR product, 2.0 µL. Thermocycling conditions with the AB 9700 are: Heat activation of 96ºC for 1 min, followed by 25 cycles of 96ºC for 10 sec, 50 °C for 5 sec, and 60ºC for 4 min. Hold at 4ºC.

b) Sequencing Reaction Cleanup: Add 1 µL of 2.2% SDS to the 10 µL sequencing reaction. Mix. Incubate at 98°C for 5 min and cool at 25°C for 10 min. Purify with the Princeton Separations Centri-Sep® Columns.

c) Data Analysis: Sequencing of sodium bisulfite treated DNA is more challenging due to the decrease in cytosine or guanine nucleotides (depending upon the DNA strand being sequenced). Sequencing software may try to compensate for the lack of "C" by increasing the detection sensitivity therefore we recommend performing electrophoresis with the Applied Biosystems 3130*xl* Genetic Analyzer or the Applied Biosystems 3730*xl* DNA Analyzer and the KB™ basecaller to avoid mis-calling. Detection of C to T conversion can be automated with SeqScape® software.

If the target DNA is of mixed methylation status, direct sequencing of the PCR product will result in a sequencing trace containing multiple peaks of T and C corresponding to non-methylated and methylated cytosine's in the original genomic DNA. Direct sequencing of PCR amplicons may not detect mixed nucleotides if one nucleotide is present in less than 10% of the sample. Analysis can be improved by cloning the PCR fragments, and then sequencing the isolated clones.

References

Boyd, V. L. and Zon, G. 2004. *Analytical Biochemistry* 326:278-280.

Clark, S. J., Harrison, J., Paul, C, L., and Frommer, M. 1994. *Nucleic Acids Res.* 22:2990.

Meissner A, Gnirke A, Bell GW, Ramsahoye B, Lander ES, Jaenisch R. Nucleic Acids Res. 2005 Oct 13;33(18):5868-77.

Product Description	Package	Part Number
methylSEQr™ Bisulfite Conversion kit. Includes methylSEQr™ Denaturation Buffer, methylSEQr™ Conversion Reagent, methylSEQr™ Purification Columns	48 reactions	4379580

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Printed in the USA, 09/2006 Publication 107PT01-01

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