Bisulfite Treatment of DNA: Perfecting the Deamination of Cytosine Into Uracil

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ABSTRACT

Sodium bisulfite can deaminate "convert" cytosine in DNA into uracil, but does not affect 5-methylcytosine. Bisulfite treatment of DNA is a prerequisite for DNA methylation analysis for many epigenetics-based studies involving methylation profiling and the quantification of methylation status. However, analytical procedures involving bisulfite treated DNA are often subject to variability due to DNA degradation, incomplete conversion, and/or low yields of DNA. We have systematically investigated the procedure of bisulfite treatment of DNA paying particular attention to the chemistries involved in the process and to conversion rates in an effort to limit variability between samples and to improve upon conventional methods. We found conventional bisulfite DNA conversion chemistries could be improved such that increased C to U conversion efficiencies could be obtained without the levels of DNA degradation typically resulting from incubation of reaction mixtures at high temperature and nonphysiological pH. Essential to this process was prohibiting the occurrence of "over-conversion" of 5-methylcytosine into uracil that can occur in some situations and reaction conditions. We found the bisulfite conversion process could be simplified and the variability between treatments kept to a minimum by coupling heat denaturation with the bisulfite conversion process and by using in-column desulphonation to clean and purify the converted DNA. This new method was found to yield an average of > 80% recovery of input DNA with > 99% C to U conversion. The method has been specifically designed to accommodate (in addition to purified DNA) biological fluids, cells, or tissue directly as the input material. This makes its application for FFPE and LCM-derived samples particularly well suited.

INTRODUCTION

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 restriction endonucleases that are designed to eliminate foreign DNA, and in higher eukaryotes DNA methylation functions in the regulation/control of gene expression (1). It has been demonstrated that aberrant DNA methylation is a widespread phenomenon in cancer and may be among the earliest changes to occur during oncogenesis (2). 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 (3). The majority of DNA methylation in mammals occurs in 5'-CpG-3' dinucleotides, but other methylation patterns do 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 twenty percent that remain unmethylated are within promoters or in the first exons of genes.

The ability to detect and quantify DNA methylation efficiently and accurately has become essential for the study of cancer, gene expression, genetic diseases, as well as many other important aspects of biology. To date, a number of methods have been developed to detect/quantify DNA methylation including: high-performance capillary electrophoresis (4) and methylation-sensitive arbitrarily primed PCR (5). However, the most common technique used today remains the bisulfite conversion method (6). This technique involves treating methylated DNA with bisulfite, which converts unmethylated cytosines into uracil. Methylated cytosines remain unchanged during the treatment. Once converted, the methylation profile of the DNA can be determined by PCR amplification followed by DNA sequencing.

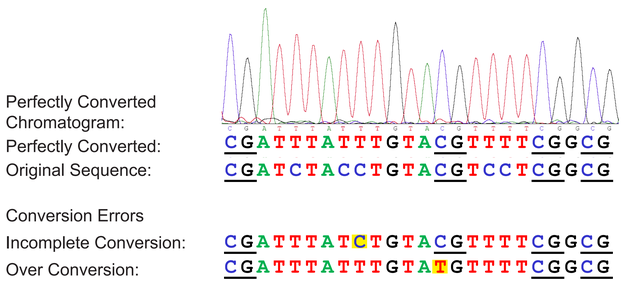
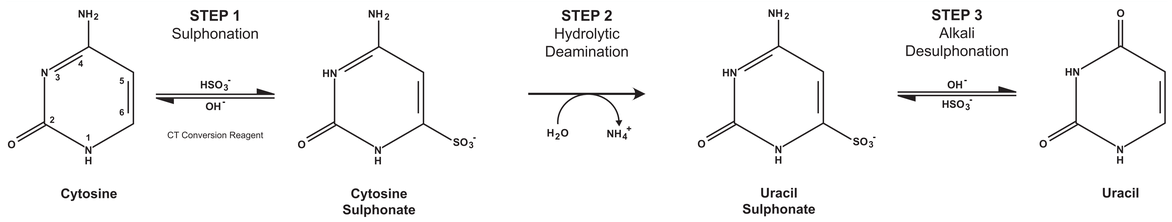


Figure 1: Methylation Profiling by Bisulfite Sequencing. A common procedure for methylation detection is bisulfite sequencing. DNA bisulfite conversion is followed by PCR amplification and sequencing display of cytosine methylation pattern. Old methods are often associated with incomplete conversions and over conversions.

Figure 2: Bisulfite Conversion of Cytosine to Uracil



RESULTS AND METHODS

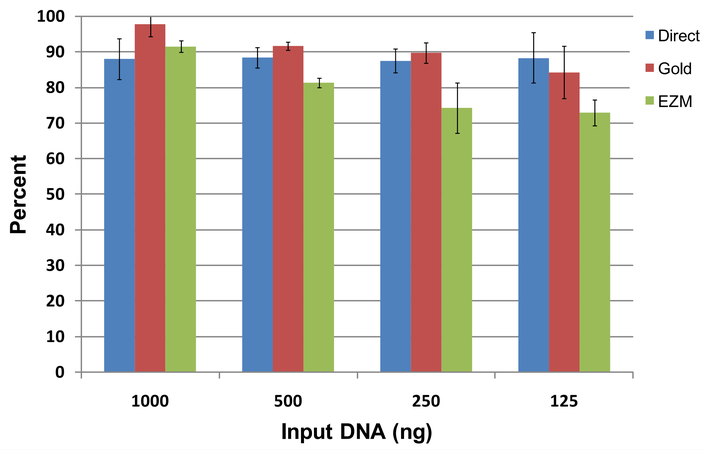


Figure 3: High Efficiency Recovery Of Input DNA. The indicated amounts of genomic DNA were processed using the EZ DNA Methylation-Direct™ (Direct), EZ DNA Methylation-Gold™ (Gold), or EZ DNA Methylation™ (EZM) Kit. The recovered DNA was quantified in quadruplet sets. Error bars represent ±1 standard deviation. All kits performed very well, with average recoveries above 70% at all DNA inputs. While the Gold and EZM kits began to decline in recovery below 500 ng of input, the Direct kit maintained greater than 80% recovery across the input spectrum from 125 to 1000 ng.

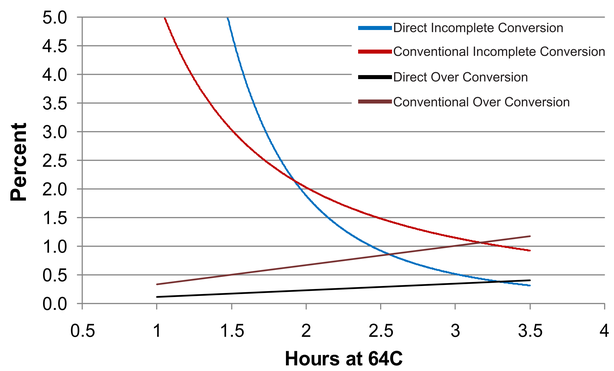


Figure 4: Improve the Conversion reaction kinetics. A plasmid universally methylated in all CpG dinucleotides was converted using either the conventional method or improved method. A time course was conducted to evaluate the conversion efficiency as the reaction progressed. All samples were processed , amplified, and sequenced. Conversion errors were tabulated for each group of sequences and graphed as a percentage of either unconverted nonmethylated cytosines (Incomplete Conversion) or converted methylated cytosines (Over Conversion). The new method improved the end point conversion results of bisulfite reaction and minimized the unwanted over-conversions.

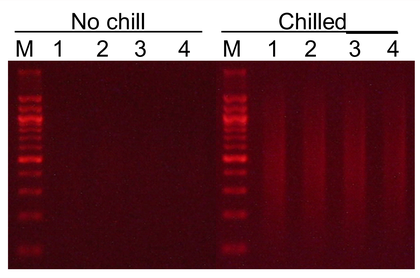


Figure 5: Simple method to analyze Bisulfite Converted DNA Using Agarose Gel. 500 ng of DNA was converted using the Direct kit. Approximately 200 ng from the elution was run on a 2% agarose gel containing ethydium bromide. A picture was taken immediately after removing from the gel tray (No chill) and another after 10 minutes in an ice bath (Chilled). After chilling the DNA appears as a smear between 100 and over 1500bp, as compared to a 100bp marker (M).

Direct gel analysis of bisulfite treated DNA is difficult because of single stranded DNA can not be stained by ethydium bromide dye. We have found that by cooling down the gel temperature, the bisulfite converted single stranded DNA can be stained and viewed under UV light to monitor the recovered DNA easily.

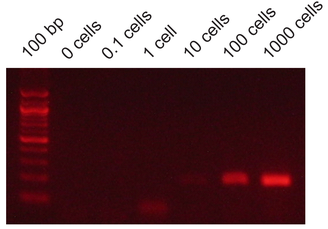


Figure 6: Sensitivity of Detection Using New Method. A suspension of human primary fibroblasts was serially diluted 1:10 in digestion buffer and proteinase K. These dilutions were then digested at 50°C for 20 minutes. 20 µl of each digestion were converted and processed using the Direct kit. PCR was conducted and results were analyzed on 2% agarose gel. The results show that the method is sensitive enough to detect as few as 10 cells.

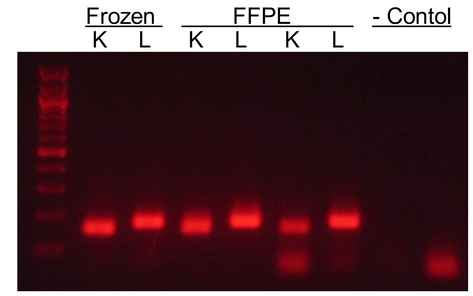


Figure 7: Bisulfite Conversion of FFPE Tissue Directly without DNA Purification Formalin fixed paraffin embedded (FFPE) and frozen mouse kidney (K) and liver (L) were processed using the Direct kit. FFPE tissues were de-paraffinized using a typical xylene protocol followed by hydration through various percentages of ethanol and water. Approximately 0.5 mg (0.5 µl) of tissue was processed for each sample. PCR was performed using 1 µl of eluted DNA, both frozen samples and FFPE samples produced expected amplicon 195 bp amplicons.

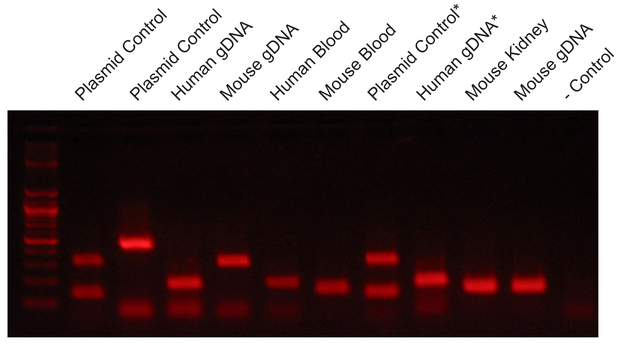


Figure 8: Direct Bisulfite DNA Conversion from Diversity Sample Sources. A variety of DNA sources were processed using the Direct kit, either the sample digestion or the purified DNA* procedures. PCRs were run with primers yielding amplicons ranging from 470 to 150bp. All DNA sources, whether tissue, blood, or purified DNA (directly or through digestion steps), gave clear PCR amplification.

CONCLUSION

1. The EZ DNA Methylation-Direct Kit provides greater than 80% DNA recovery.
2. Over 99.8% Conversion efficiency can be achieved with less than 0.5% overconversion.
3. Recovered DNA is only moderately degraded.
4. Sensitivity of detection reaches down to the 10 cell range when using cellular inputs.
5. Fixed tissue samples can be analyzed, without prior purification.
6. A wide range of DNA sources can be used as input materials from purified plasmid to whole blood and fixed tissues.
7. Simple method was developed to view converted DNA using conventional agarose gel analysis.

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