Direct Quantitative Bisulfite Sequencing Using Tag-modified Primers and Internal Normalization

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Abstract. For the investigation of DNA methylation patterns, bisulfite conversion of the DNA followed by polymerase chain reaction (PCR) amplification and sequencing of the region of interest is the method of choice when information at single CpG site resolution is desired. In this study, a simple method for direct quantitative bisulfite sequencing based on the Sanger method is shown to be usable for the accurate analysis of single CpG sites. This method is based on the usage of tag-modified primers to obtain an internal normalization signal within the PCR product.

DNA methylation alterations are among the most promising candidates for cancer biomarker research. Sequencing of bisulfite DNA is the preferred method when detailed information on the methylation pattern of a given template is desired at single CpG resolution. Currently, bisulfite sequencing based on the chain-termination reaction developed by Sanger and co-workers is among the most commonly used methods for methylation analysis (1, 2). Quantification of methylation is usually carried out by cloning of the polymerase chain reaction (PCR) product followed by sequencing a number of individual clones. Therefore, the resolution directly correlates with the number of analyzed clones, which is time consuming and expensive when a high resolution is desired. A more cost-efficient and faster approach is achieved by direct sequencing of the PCR product without any cloning steps (3). This approach provides a quantitative measure of the degree of methylation at a particular target site by comparing cytosine and thymine signals. However, it is susceptible to basecaller artefacts and therefore limited in its resolution. Other technologies, such as pyrosequencing (4, 5), also allow for the quantitative analysis of single CpG sites, but the required instruments are less commonly available in many laboratories.

Thus, inexpensive and simple methods for the accurate quantification of single CpG sites with direct bisulfite sequencing using standard laboratory equipment, such as capillary electrophoresis instruments, would offer a significant improvement for DNA methylation analysis.

Here, an improved method for quantitative bisulfite sequencing of single CpG sites based on the Sanger method is presented. Accurate quantification is achieved by the incorporation of a domain into the PCR product, which is subsequently used for signal normalization (6).

Materials and Methods

Unmethylated DNA was prepared by multiple displacement amplification (MDA), a genome-wide amplification method (7). For the preparation of DNA mixtures with defined methylation ratios, a portion of the MDA amplificate was treated with SssI methyltransferase (New England Biolabs, Ipswich, MA, USA) in the presence of S-adenosyl-methionine, according to the manufacturer’s instructions, and mixed with the unmethylated amplificate to give mixtures representing 0, 5, 10, 15, 25, 50, 75 and 100% methylation. Two micrograms of each of these mixtures were bisulfite treated using the EpiTect® kit (Qiagen, Hilden, Germany). The resulting bisulfite DNA concentration was determined by UV spectrophotometry using a Nanodrop® ND-1000 spectral photometer (Nanodrop Technologies, Wilmington, DE, USA).

PCR amplification was carried out at 25 μl scale (10 ng DNA, 1 U HotStar Taq polymerase (Qiagen), 1 x PCR buffer (Qiagen), 0.2 mM each dNTP (Fermentas, Burlington, Canada), 0.5 μM both primers (reverse primer: aggtgTTCTAATCCTCCTTTCCACAATAA, forward primer: GTAGGGGAGGGAAGTAGATGTT; the normalization domain is indicated using lower case letters). Incubation was performed using the following temperature profile: 15 min at 95˚C and 45 cycles with 20 s at 95˚C, 45 s at 58˚C and 30 s at 72˚C. Sequencing was carried as previously described (6). Raw data were extracted from the ABI sequencing electropherograms using BioEdit 6.0.7 software (Ibis Pharmaceuticals, Inc., Carlsbad, CA, USA). The trace containing the methylation information was visualized and the normalization signal identified. Electropherograms were aligned using the normalization signals in the primer tails. The
signals were normalized by integrating the signals of the normalization domain and by dividing each data point of the electropherogram by this reference value. Thirty data points surrounding each identified peak maximum within each trace were integrated to obtain the methylation score for the respective CpG.

Results

The principle of the signal normalization is shown in Figure 1. The cytosine traces containing the methylation information from five different methylated DNA mixtures are shown before (Figure 1A) and after normalization (Figure 1B). The normalization signal was derived from the three cytosines, which were incorporated into the PCR product. After normalization, the peak areas (and heights) at the CpG sites of interest were found to correlate with the relative methylation of the template DNA.

The methylation of five single CpG sites within the promoter region of the PITX2 gene was analyzed in order to demonstrate the power of the direct quantitative bisulfite sequencing method. For this purpose, DNA mixtures of unmethylated and methylated DNA were prepared and analyzed. The paired-like homeodomain 2 (PITX2) gene was chosen since it represents one of the best validated DNA methylation biomarkers in various types of cancer (8-11, 13, 14). The results of the analysis of five CpG sites within the PITX2 gene are illustrated in Figure 2. The areas of the normalized methylation signals correlated well with the methylation of the applied DNA mixture for each of the five CpG sites (Figure 2). All seven mixtures (0, 5, 10, 25, 50, 75 and 100%) were clearly distinguishable.

Discussion

DNA methylation biomarkers have shown promising results in the clinical management of cancer. Most methodologies used to assess the methylation status of a certain gene locus are based on preceding bisulfite conversion of the DNA. Bisulfite treatment of the template DNA leads to deamination of unmethylated cytosines to uracil, leaving only methylated cytosines unaltered (15). Thus, the converted DNA only contains cytosines at positions which were originally methylated.

Amplification of the converted DNA using reverse primers containing guanosines at their 5’ end leads to the incorporation of cytosines into the PCR product. These additional cytosines at the 3’ end of the PCR product are present in each molecule of the PCR product and therefore
In this study, it has been shown that the presented method for direct quantitative bisulfite sequencing based on the Sanger method is a simple, affordable and powerful tool for the accurate quantification of methylation of single CpG sites using standard laboratory equipment, such as capillary electrophoresis instruments.

References


Received September 27, 2016
Revised October 13, 2016
Accepted October 14, 2016