Methylation Analysis by Restriction Endonuclease Digestion and Real-Time PCR

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DNA methylation is a highly characterized epigenetic modification of the human genome that is critical for normal cell processes, including tissue regulation, genetic expression development, genomic imprinting, X-chromosome inactivation, and DNA repair. DNA methylation in humans occurs almost exclusively at the C5 position of CpG dinucleotides in genomic promoter regions. Alterations in DNA methylation cause developmental diseases, such as genetic imprinting defects, cancer, and potentially a wide range of adult-onset chronic diseases. Therefore, sensitive techniques that detect methylation are critical for epigenetic research, clinical diagnostics, cancer prognosis, and therapeutics (1, 2).

Methylation-specific PCR is a commonly used rapid technique for methylation screening that replaces the laborious Southern blot assay, the standard procedure on which most DNA analysis is based. Methylation-specific PCR and other PCR-based methylation methods that use bisulfite-treated DNA as a template are generally accepted as the most analytically sensitive and specific techniques for analyzing DNA methylation at a single locus. The protocol described by Frommer et al. has been widely used for sodium bisulfite modification, and a variety of commercial kits are available for this purpose (3). Sodium bisulfite demethylates unmethylated cytosine to uracil, and the methylation is accessed by the PCR with either methylation-specific primers or methylation-independent primers (MIPs).4 Methylation-specific primers amplify either methylated or unmethylated alleles. These primers are highly sensitive and able to detect the presence of a methylated allele at a frequency as low as 0.1% in an unmethylated population (4). PCR with methylation-specific primers is not quantitative, however. Alternatively, the MethyLight method is a sensitive, fluorescence-based real-time PCR technique capable of quantifying DNA methylation at a specific locus (5, 6). MethyLight can detect completely methylated or unmethylated alleles but is oblivious to partially methylated CpGs. Considering the random conversion of cytosine to uracil, it is possible that a subset of DNA copies have a substantially lower conversion rate and that some promoter regions might prove more prone to incomplete conversion than others, thereby leading to the disregard of several partially methylated alleles. Therefore, MethyLight, despite being a quantitative assay, cannot distinguish different methylation patterns. MIP-PCR uses primers designed for universally amplifying proportional methylated and unmethylated alleles that include a number of CpG sites. Because of their GC content, the methylated and unmethylated alleles are distinguished by high-resolution melting analysis, which exploits differences in thermal stability (7). This PCR has a tendency to amplify unmethylated DNA, because of the low GC content after bisulfite modification of the DNA. The sensitivity of MIP-based methods is relatively low, but it can be increased by introducing CpG sites into the primers or by using oligonucleotide blockers. Nonetheless, these assays can measure methylation quantitatively and estimate the mosaic situation. Several other MIP-based methods also have been successfully used for methylation analysis, such as methylation-sensitive single-nucleotide primer extension and combined bisulfite restriction analysis (8).

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) uses multiple probes that are hybridized to their targeted methylation sites. The methylation sites are recognized by a methylation-sensitive restriction enzyme (HhaI), and the probe pair is ligated in the ligation reaction (9). This method not only permits simultaneous analysis of multiple methylation sites but also allows quantification of copy number changes by comparing target-specific probes to the control probes (10).

Bisulfite Sanger sequencing, considered the gold standard for methylation analysis, provides information about individual CpG sites. In addition to requiring laborious processes to implement, the Sanger sequencing approach has a sensitivity for detecting low-level mosaicism that is limited to 20%. An attractive alternative is pyrosequencing. Pyrosequencing is
a sequencing-by-synthesis technology that luminesmetrically detects pyrophosphate released via an enzyme cascade during synthesis. With a read length of 25–30 bp, pyrosequencing methylation analysis relies on bisulfite conversion and yields quantitative information on single CpG sites (11).

Although the bisulfite modification–based methods described above perform acceptably in many scenarios, their use of multiple chemical reactions to convert cytosine to uracil requires a purification process after the conversion, thus leaving room for contamination during the many steps. Therefore, there is a need for a better method that is rapid, highly sensitive, and cost-effective and that has both a low false-negative rate and a low risk of contamination. In this issue of Clinical Chemistry, von Kanel et al. describe a novel quantitative real-time PCR assay that detects DNA methylation in a 1-step, single-tube reaction (12). This PCR uses untreated genomic DNA as a template, thus eliminating the use of a bisulfite-conversion process and its considerable drawbacks, which include incomplete bisulfite reactions (13). The advantages of the approach of von Kanel et al. include the use of a single-tube reaction that combines a rapid methylation-sensitive restriction endonuclease digestion and a quantitative PCR (qPCR), thus decreasing both hands-on time and possible cross-contamination. It also provides a short turnaround time (an important factor from a diagnostic laboratory’s perspective), a relatively low cost, the flexibility to design a real-time PCR-based assay, and the use of a model for correcting results obtained with low-quality DNA, such as that in formalin-fixed, paraffin-embedded tissue samples, which are usually degraded or possess some PCR inhibitors.

The single-tube reaction described by von Kanel et al. is performed in a glass capillary–based LightCycler instrument (Roche Applied Science) within 90 min by incubating with the methylation-sensitive FastDigest® HpaII enzyme (Fermentas Life Sciences) at 37 °C for 10 min to digest the genomic DNA template and then at 5 min at 95 °C to inactivate the HpaII enzyme and activate the Taq polymerase. This step is followed by 40 cycles of a real-time PCR reaction at an annealing temperature of 60 °C (15 s) for both the SNRPN5 (small nuclear ribonucleoprotein polypeptide N) locus (target) and the CFTR [cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7)] locus (control for DNA digestion and reference for copy number analysis). Performing the sham reactions allows measurement of the methylation status (as a percentage) with the default second derivative maximum method of the LightCycler for determining the quantification cycles (Cqs) with and without the HpaII digestion. In addition, the ΔΔCq method (14) is used to evaluate the copy number of the SNRPN promoter by obtaining the Cqs from the sham reactions of the patient samples and the calibrator (DNA with a known copy number status). This copy number analysis is independent of DNA digestibility. With this 1-step, single-tube approach, von Kanel et al. have validated a set of blinded samples (from 12 patients with Prader–Willi syndrome, 12 patients with Angelman syndrome, and 11 unaffected individuals) and found 100% concordance with the previously obtained genotypic data (by methylation-specific PCR followed by denaturing HPLC and microsatellite analysis) for both methylation status and copy number change.

To further minimize the impact of low DNA quality on the quantitative assessments caused by the digestion bias, this study also investigated an additional 14 DNA samples with reduced digestibility and provided a bias-correction model, with the CFTIR locus used as a control [i.e., CSML3 = MM_{SNRPNpat} + MM_{CFTRpat} × ((MM_{SNRPNcal/MM_{CFTRcal}}) × (1 − CSML3/100)]. This model corrects the measured percentage of methylation to more closely match the theoretical values (0%, 50%, 100%) when DNA samples of both good quality and reduced digestibility are used. Use of this equation requires that DNA with a known methylation status be included in the experiment as a calibrator. Furthermore, this study also used the linear relationship observed between the theoretical and measured DNA methylation levels during assay validation to determine the percentage of mosaicism in the tested samples from patients with Prader–Willi syndrome and Angelman syndrome.

Considering this report’s thorough analysis of parameters that may affect the performance of real-time PCR assays, including different types of DNA samples, several outstanding issues remain to be addressed. First, in addition to initial DNA quality and the method used for DNA extraction, what often affects the robustness (performance) and the throughput of a real-time qPCR assay is the selection of the proper reference gene(s) for better quantitative assessment. When this method is expanded to analyze multiple methylated loci simultaneously, it is necessary to determine whether the same single reference gene (CFTR) can be used or whether a “reference panel” of genes is needed for better quantitative assessment. MS-MLPA certainly requires a relatively longer hands-on time (estimated at 10 min for the first day of hybridization setup and 45 min on the second day to finish digestion/ligation be-

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5 Human genes: SNRPN, small nuclear ribonucleoprotein polypeptide N; CFTR, cystic fibrosis transmembrane conductance regulator (ATP-binding cassette sub-family C, member 7).
fore the PCR and fragment analysis), but it analyzes multiple loci simultaneously and uses multiple built-in reference genes in the same reaction/sample/run, making the data-analysis portion of the assay relatively easy and robust for assessing both DNA methylation status and copy number change. Second, although this 1-step, single-tube qPCR assay has been proposed for measuring the mosaicism percentage, it has not addressed fully the calculation of mosaicism. A larger sample size is needed to better establish the legitimacy of this proposed feature.

Ultimately, the method von Kanel et al. have developed provides a rapid, efficient, and accurate quantitative assessment of both DNA methylation status and copy number change at a single locus, with the added benefit of determining the percentage of mosaicism. Quantification of the methylation sites can be achieved with real-time PCR, and a closed-tube assay avoids potential cross-contamination. This novel 1-step, single-tube qPCR method fulfills the requirements of being a more cost-effective, efficient, and thorough assay, which can be readily implemented in a diagnostic setting to improve patient services.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

Authors’ Disclosures of Potential Conflicts of Interest: No authors declared any potential conflicts of interest.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

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