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 deaminates unmethylated cytosine to uracil, and the methylation is accessed by the PCR with either methylation-specific primers or methylation-independent primers (MIPs). Methylated DNA is ligated in the ligation reaction that are hybridized to their targeted methylation sites. 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.
a sequencing-by-synthesis technology that lumino-
metrically detects pyrophosphate released via an en-
zyme cascade during synthesis. With a read length of
25–30 bp, pyrosequencing methylation analysis relies
on bisulfite conversion and yields quantitative infor-
amation on single CpG sites (11).

Although the bisulfite modification–based meth-
ods described above perform acceptably in many sce-
narios, their use of multiple chemical reactions to con-
vert cytosine to uracil requires a purification process
after the conversion, thus leaving room for contamina-
tion 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 incom-
plete 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®
Hpall 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 acti-
ivate the Taq polymerase. This step is followed by 40
cycles of a real-time PCR reaction at an annealing tem-
perature of 60 °C (15 s) for both the SNRPN5 (small
nuclear ribonucleoprotein polypeptide N) locus (tar-
get) and the CFTR [cystic fibrosis transmembrane con-
ductance 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 deriv-
ative maximum method of the LightCycler for deter-
mining the quantification cycles (Cq) with and with-
out the HpaII digestion. In addition, the ΔΔCq method
(14) is used to evaluate the copy number of the SNRPN
promoter by obtaining the Cq from the sham reactions
of the patient samples and the calibrator (DNA with a
known copy number status). This copy number analy-
sis is independent of DNA digestibility. With this
1-step, single-tube approach, von Kanel et al. have val-
ified a set of blinded samples (12 patients with Prader–Willi
syndrome, 12 patients with Angelman
syndrome, and 11 unaffected individuals) and found
100% concordance with the previously obtained geno-
typic 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 qual-
ity on the quantitative assessments caused by the ineffi-
cient digestion, this study also investigated an addi-
tional 14 DNA samples with reduced digestibility and
provided a correction model, with the CFTR locus
used as a control [i.e., CSMLOa = MMNSRPNpat −
MMCFTRpat × (MMNSRPNcal/MMCFTRcal) × (1 −
CSMLOa/100)] [see Eq. 5 in the report by von Kanel et al.
(12)]. This model corrects the measured percentage
of methylation to more closely match the theoretical val-
ues (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 relation-
ship observed between the theoretical and measured
DNA methylation levels during assay validation to de-
termine the percentage of mosaicism in the tested
samples from patients with Prader–Willi syndrome and
Angelman syndrome.

Considering this report’s thorough analysis of pa-
rameters 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 robust-
ness (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

5 Human genes: SNRPN, small nuclear ribonucleoprotein polypeptide N; CFTR,
cystic fibrosis transmembrane conductance regulator (ATP-binding cassette
sub-family C, member 7).
min on the second day to finish digestion/ligation before 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.

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