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Novel Assay for Quantitative Analysis of DNA Methylation at Single-Base Resolution

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BACKGROUND: The DNA methylation profile provides valuable biological information with potential clinical utility. Several methods, such as quantitative methylation-specific PCR (qMSP), have been developed to examine methylation of specific CpG sites. Existing qMSP-based techniques fail to examine the genomic methylation at a single-base resolution, particularly for loci in gene bodies or extensive CpG open seas lacking flanking CpGs. Therefore, we established a novel assay for quantitative analysis of single-base methylation.

METHODS: To achieve a robust single-base specificity, we developed a PCR-based method using paired probes following bisulfite treatment. The 6-carboxyfluorescein- and 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein-labeled probes conjugated with minor groove binder were designed to specifically bind to the methylated and unmethylated allele of targeted single CpGs at their 3′ half regions, respectively. The methylation percentage was calculated by values of methylation / (methylation + unmethylation).

RESULTS: In the detection of single CpGs within promoters or bodies of 4 human genes, the quantitative analysis of the single-base methylation assay showed a detection capability in the 1 to 1:10 000 dilution experiments with linearity over 4 orders of magnitude ($R^2 = 0.989–0.994$; all $P < 0.001$). In a cohort of 10 colorectal cancer samples, the assay showed a comparable detection performance with bisulfite pyrosequencing ($R^2 = 0.875–0.990$; all $P < 0.001$), which was better than conventional qMSP methods normalized by input control reaction ($R^2 = 0.841$ vs 0.769; $P = 0.002$ vs 0.009).

CONCLUSIONS: This assay is highly specific and sensitive for determining single-base methylation and, thus, is potentially useful for methylation-based panels in diagnostic and prognostic applications.

The cytosine-5 DNA methylation profile of a cancer specimen is considered a valuable source of biological information with potential clinical utility *(1)*. The cytosine-5 DNA methylation mainly occurs in the context of CpG dinucleotides (a cytosine base followed immediately by a guanine base) in vertebrates, and transcriptional inactivation of CpG island (CGI)[4]-containing promoters of tumor suppressor genes by hypermethylation has been well-documented in many human cancers *(1–3)*. However, the CGIs within promoters represent only a small fraction of the methylome, and the low CpG density regions located in gene bodies represent the most conserved targets of DNA methylation among eukaryotes *(4)*. In addition, gene body methylation may serve as a potential mechanism of RNA alternative splicing regulation *(5, 6)* and confine transcription initiation, thus preventing aberrant transcripts *(4, 7)*. Thus, the biological role of the methylation in a low CpG density region remains to be determined for diagnostic or prognostic markers and potential therapeutic targets in cancer *(8)*.

Considering the clinical significance of analyzing the genomic methylation, a number of methods have been developed. Methylation-specific PCR (MSP) is an end point analysis technique *(9, 10)*, and MethyLight [quantitative MSP (qMSP)] has been introduced to draw quantitative conclusions *(11, 12)*. However, the existing DNA methylation assays are mostly used to determine the local mean of multiple CpG sites within the high CpG density region, such as CGI, based on the assumption that all the local CpG sites are fully methyl-

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[4] Nonstandard abbreviations: CGI, CpG island; (q)MSP, (quantitative) methylation-specific PCR; QASM, quantitative analysis of single-base methylation; 6FAM, 5` fluorescent reporter dye 6-carboxyfluorescein; VIC, 2`-chloro-7`phenyl-1,4-dichloro-6-carboxy-fluorescein; MGB, minor groove binder; NFQ, nonfluorescent quencher; CT, cycle threshold; EPIC, Illumina MethylationEPIC.

[5] Human genes: *FHIT,* fragile histidine triad; *SGIP1,* SH3 domain GRB2 like endophilin interacting protein 1; *KAZN,* kazrin, periplakin interacting protein; *ACTB,* actin beta; *CDO1,* cysteine dioxygenase type 1.

ated or unmethylated. Thus, the oligos are designed to cover CpG dinucleotides as much as possible to increase analytical sensitivity and specificity. Previous studies applied an improved MethyLight assay to determine single-base methylation in patient samples using various strategies *(13–16)*, but the studies lacked systematic evidence or optimized methods. Therefore, it is essential to have an optimum solution to low CpG density region, or the condition that adjacent CpG sites are not comethylated, which has been shown to be common in recent genome-wide analysis, even in CGIs *(17)*. Bisulfite pyrosequencing or targeted bisulfite sequencing can be applied in this condition, but these techniques are labor-intensive, time-consuming, or lacking cost-effectiveness for utility in cohort studies or clinical practice. The MethyLight assay using ALU repeat-based input control reaction has been widely used to determine the methylation of CGIs in batch samples *(18, 19)*. The other limitation of MethyLight is the need for an input control and fully methylated DNA as a reference control to calculate the methylation percentage. However, an input control may introduce stochastic errors, and a fully methylated DNA is not easy to find quality control materials.

To investigate the methylation status of single CpGs, we translated the TaqMan genotyping principle to bisulfite-treated DNA with the single-nucleotide polymorphism being the CpG after bisulfite treatment. We developed an assay for the quantitative analysis of single-base methylation (QASM).

We designed and applied this assay to examine the methylation percentages of 3 loci in bodies of 3 genes. We evaluated the QASM measurements on tumor DNA samples by the comparison with accurate bisulfite pyrosequencing. Additionally, we exploited the methylation/unmethylation ratio to calculate methylation percentage and, thus, avoided an independent PCR reaction to control input DNA concentrations. We also developed a computational method that permits one to determine the percentage of methylated alleles without reference to the fully methylated control sample.

Materials and Methods

SAMPLE COLLECTION

Ten snap-frozen tumor tissue samples were obtained from 10 patients with primary colorectal adenocarcinoma. All samples used for the methylation arrays were reviewed by a gastrointestinal pathologist to confirm the diagnosis and ensure that the cancer samples were >60% tumor epithelium. Among the patients, 7 had stage I tumors and 3 had stage II tumors. This study was approved by the Institutional Review Board of Sun Yat-sen University, Guangzhou, China. Informed consent was obtained from patients to have their tissue used for research purposes.

DNA ISOLATION AND BISULFITE CONVERSION

Genomic DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, 51306), and bisulfite was modified using the EZ DNA Methylation Kit (Zymo Research, D5002). Sodium bisulfite conversion of genomic DNA was performed as previously described *(10, 20)*.

QASM ASSAY

After sodium bisulfite conversion, the methylated CpGs are refractory to bisulfite and remain as CpG on both DNA strands, whereas an unmethylated CpG is deaminated to a TpG after bisulfite conversion; thus, the use of C/T-specific probes carrying 2 different fluorophores permits methylation determination in the same tube. The bisulfite-converted genomic DNA was amplified using locus-specific PCR primers flanking a pair of oligonucleotide probes, each labeled with a 5′ fluorescent reporter dye 6-carboxyfluorescein (6FAM) or 2′-chloro-7′phenyl-1,4-dichloro-6-carboxy-fluorescein (VIC), and a 3′ quencher dye minor groove binder–nonfluorescent quencher (MGB-NFQ) (Fig. 1). The MGB groups were conjugated with 3′-quencher to allow shorter probes to be used and have a high sensitivity and specificity to single-base mismatch *(21)*. The 5′ to 3′ nuclease activity of *Taq*DNA polymerase cleaved the probe and released the reporter, whose fluorescence could be detected by the Applied Biosystems QuantStudio 7 Flex Real-Time PCR System. Initial template quantity could be derived from the cycle threshold (CT) value at which the fluorescent signal crossed a threshold in the PCR reaction *(22)*. Serial dilutions of a control sample were included on each plate to generate a standard curve. We performed the PCR with a final reaction mixture of 20 μL consisting of 500 nmol/L each primer; 150 nmol/L probe; 200 nmol/L each dATP, dCTP, dGTP, and dTTP; 2.25 mmol/L $MgCl_2$; 0.75 U of HotStarTaq enzyme; 1× PCR buffer; and bisulfite-converted DNA or reference DNA at the following conditions: 95 °C for 15 min, followed by 50 cycles at 94 °C for 30 s, 58 °C for 1 min, and 72 °C for 1 min. The protocol file in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol65/issue5 contains detailed information about the QASM assay.

METHYLATION CALCULATIONS

Because neither the input control nor fully methylated reference DNA was applied to the QASM assay, the methylation percentages of specific CpG sites were calculated differently from the previous report *(18)*. The methylation percentage of each sample was previously calculated by $(\text{methylation/input control})_{\text{test sample}}/(\text{methylation/input control})_{\text{fully methylated reference sample}} \times 100$, whereas in the QASM assay it was equal to methylation/(methylation + unmethylation) × 100, and we used

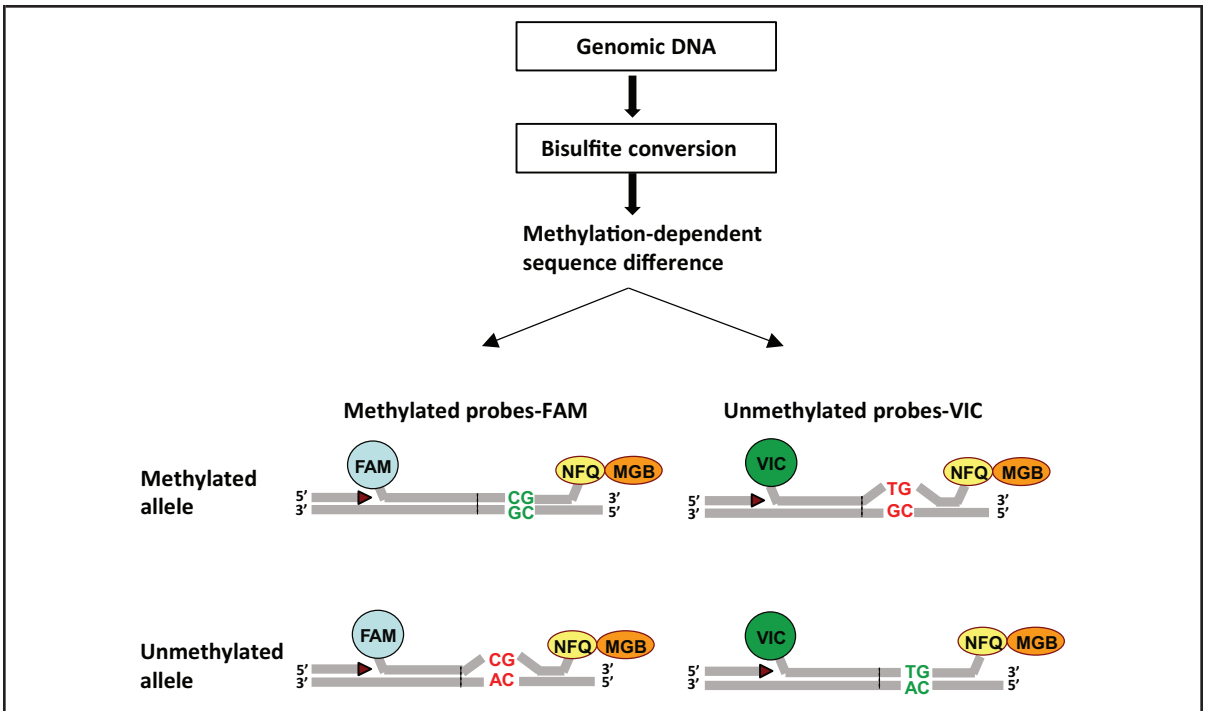

Fig. 1. Strategy of QASM assay for single CpGs.

The sodium bisulfite treatment of genomic DNA created methylation-dependent sequence differences. A FAM-labeled MGB probe was used to specifically hybridize to the sequence derived from the methylated allele (CpG), and a VIC-labeled probe was used to bind to the sequence generated from the unmethylated allele (TpG). Both methylated and unmethylated alleles are amplified in the same reaction tube with 1 primer pair, and the sequence discrimination occurs only at the level of the fluorogenic probe hybridization.

this formula to calculate the methylation percentage: $100/(1 + 1/2^{-\Delta CT})$, $\Delta CT = CT_{methylation} - CT_{unmethylation}$.

PRIMER AND PROBE SEQUENCES

Three sets of PCR primers and probes, designed specifically for bisulfite-converted DNA sequences, were used for fragile histidine triad (*FHIT*,[5] NM_002012), Src homology 3 domain growth factor receptor-bound protein 2-like endophilin interacting protein 1 (*SGIP1*, NM_032291), and kazrin periplakin interacting protein (*KAZN*, NM_201628). The primers and probes used in the QASM assay are listed in Table 1. Fig. 1 and Text 1 in the online Data Supplement provide the designed oligos hybridized to the flanking sequence of the 3 loci. The protocol file in the online Data Supplement provides information about how to design primers and paired probes that could specifically determine single-base methylation.

BISULFITE PYROSEQUENCING ASSAYS

PCR amplification was performed using 50 μL of reaction mixture containing 0.5 μmol/L each of the biotinylated forward and reverse primers (Table 1), 1 U of *Taq* polymerase, 2.0 mmol/L $MgCl_2$, and 200 μmol/L each of dNTP, and 10 ng of bisulfite-converted template DNA on the PCR System (Applied Biosystems, Verity 96well). For the pyrosequencing reaction, the PCR products were purified and isolated. The DNA strands were then released into a mix containing 25 μL of annealing buffer with 0.3 μmol/L of sequencing primers (Table 1). Pyrosequencing reactions were performed in the PyroMark Q96 ID (Qiagen), and CpG site quantification was performed using the PyroMark CpG Software 1.0.11. Methylation percentages were calculated by using the following formula: height of the C/G peak / (height of the C/G peak + height of the T/A peak) × 100.

Results

STRATEGY BEHIND THE QASM ASSAY

Sodium bisulfite treatment of genomic DNA converts unmethylated cytosines to uracil, whereas methylated cytosine residues remain unaffected. This modification creates methylation-dependent sequence differences in the genomic DNA. In the current QASM assay, we used 2 different fluorescence-labeled MGB-conjugated

Table 1 Primers and paired probes used to test QASM assay.

Primers and oligos	Sequence 5′ to 3′	T_m	Amplicon size
QASM assay			
SGIP1-forward	TTTAGAGATTATGGAGTTTTTTAGAAGGGTT	59.4	113
SGIP1-reverse	TCATAAACTACCCTACCCTTTTCTTCCTT	60.9	
SGIP1-M FAM probe	6FAM-GTTTTGGGGGATTTACGA-MGB-NFQ	69.0	
SGIP1-U VIC probe	VIC-GTTTTGGGGGATTTATGA-MGB-NFQ	69.0	
FHIT-forward	ATTCGATGAGTTTATTGTATTGTTTATTTATTTG	59.6	127
FHIT-reverse	TCATACCCCAAACATAAACATCATACA	59.3	
FHIT-M FAM probe	6FAM-TGATTTCGGGGTGTATA-MGB-NFQ	64.0	
FHIT-U VIC probe	VIC-TTTGATTTTGGGGTGTAT-MGB-NFQ	64.0	
KAZN-forward	TTGGAAGAGGAGTTATAATGTAGGATGTTG	60.9	96
KAZN-reverse	CCTCTTAAATCCCTTCAAACCTTTTCTTAT	59.6	
KAZN-M FAM probe	6FAM-TTAAGGTAGGTAAGCGTGG-MGB-NFQ	66.0	
KAZN-U VIC probe	VIC-AAGGTAGGTAAGTGTGGTGT-MGB-NFQ	66.0	
CDO1-forward	GGAGGATGAATTTTATAGATTTGAATTTGATTTG	59.5	93
CDO1-reverse	CACCCCCAAACCCCTAACA	59.1	
CDO1-M FAM probe	6FAM-CAATAAATCCGAAATC-MGB-NFQ	66.0	
CDO1-U VIC probe	VIC-CAATAAATCTGAAATC-MGB-NFQ	65.0	
ALU-C4 control reaction			
ALU-C4-forward	GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA	59.1	98
ALU-C4-reverse	ATTAACTAAACTAATCTTAAACTCCTAACCTCA	59.4	
ALU-C4-probe	NED-CCTACCTTAACCTCCC-MGB-NFQ	70.0	
Bisulfite pyrosequencing assay			
SGIP1-forward	TGAGTTTTTTTGAAGGGAATTAAGGG	60.9	89
SGIP1-reverse	ACCTAACTACTCTCCAAAAACCATAAA	60.1	
SGIP1-sequencing primer	ACCCAACCCTAAAAAAT	46.2	
FHIT-forward	TGATAAATTTGTTAAAAGAAAGAGGAAGTA	58.4	168
FHIT-reverse	CCTATAAAACAAACCCACATATACAC	58.1	
FHIT-sequencing primer	CCCACATATACACCC	44.6	
KAZN-forward	GGGATTTTAATTGTTATTGGAGAATAGG	59.5	250
KAZN-reverse	AAAACATTAATTCCCAAATACTTAAATTCT	58.6	
KAZN-sequencing primer	CAAAAACACACCAAAAC	44.8	
CDO1-forward	TTATAGATTTGAATTTGATTTGTGTG	53.6	79
CDO1-reverse	CCCCCAAACCCCTAACAAC	57.7	
CDO1-sequencing primer	AATTTGATTTGTGTGTGTAT	43.8	

TaqMan® probes during PCR amplification to achieve single-base discrimination. Both methylated and unmethylated alleles were amplified in the same reaction tube with 1 primer pair, and the sequence discrimination occurred only at the level of the fluorogenic probe hybridization and was based on the differential annealing of the perfectly matched vs mismatched oligonucleotides (Fig. 1). We used a FAM-labeled MGB probe that specifically hybridized to the sequence derived from the methylated allele and a VIC-labeled probe that bound to the sequence generated from the unmethylated allele. The single-base mismatch in the 3′ half region (MGB region) of the probe has a ΔT_m up to 17 °C compared with the perfect match *(21)*. This allowed us to design the primers and paired probes to make just the probes cover single CpG dinucleotide and, thus, make it possible to measure the methylation of the single CpGs. Using the methylation percentage formula described earlier, the ratio between the signals from the methylated probe and the unmethylated probe would provide a precise quanti-

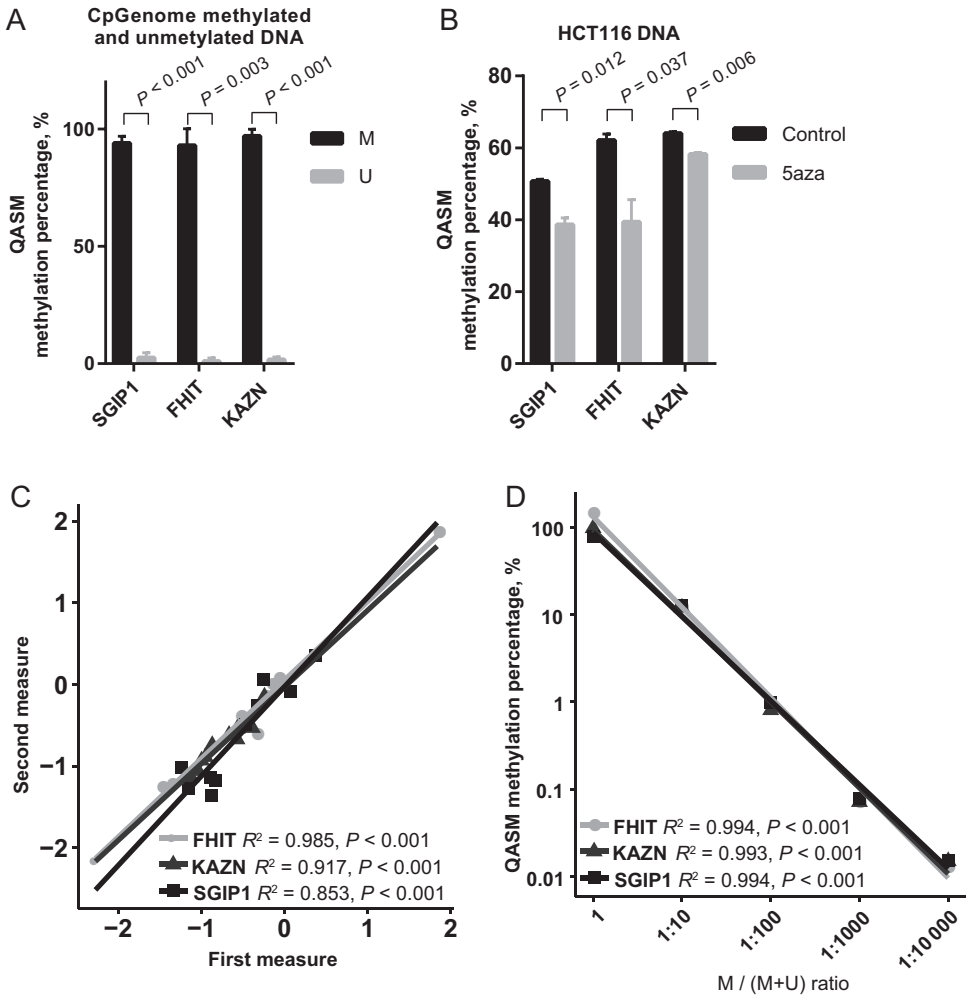

Fig. 2. Analytical specificity, sensitivity, and reproducibility of QASM assay.

(A), Use of the QASM assay to examine the methylation of SGIP1, FHIT, and KAZN in the universal fully methylated and unmethylated DNA. (B), Methylation of SGIP1, FHIT, and KAZN was expectedly decreased when HCT116 cells were treated with the DNA demethylation agent 5-aza-2`-deoxycytidine (5-aza). (C), Reproducibility of the assay on heterogeneous tumor samples. Data represent 3 independent experiments. Data are mean (SD); *P* values were calculated using Student *t*-test. (D), Serial dilution in 10-fold increments up to 1:10 000 with fully unmethylated human genomic DNA. The methylation percentage of SGIP1 was obtained for each dilution and plotted against the dilution factor.

fication for the prevalence of methylated molecules at this locus. A reaction for the amplification of control genes, such as *ACTB (11)*, and a consensus sequence of ALU-repetitive elements (ALU-C4) *(18)* to reflect and control the amount and integrity of input genomic DNA was not needed, and also a reaction for CpGenome universal fully methylated DNA sample to calculate the methylation percentage of each sample was not necessary.

VALIDATION OF THE QASM ASSAY

The single CpG sites within open seas located in the gene body of *SGIP1*, *FHIT*, and *KAZN* genes (see Text 1 in the online Data Supplement) were selected to validate the assay in the human colorectal cell line HCT116 and CpGenome universal methylated and unmethylated DNA (Millipore S7821 and S7822).

We first examined the analytical specificity of the primers and paired methylated and unmethylated probes (Table 1). The universal methylated DNA yielded a calculated methylation percentage reaching 100%, consistent with its fully methylated status. In contrast, the universal unmethylated DNA had a calculated methylation percentage close to zero (Fig. 2A). In addition, the QASM assay showed that the methylation of *SGIP1*

($P = 0.012$), *FHIT* ($P = 0.037$), and *KAZN* ($P = 0.006$) was expectedly and significantly decreased when HCT116 cells were treated with the DNA demethylation agent 5-aza-2′-deoxycytidine (Fig. 2B), showing that the QASM assay could discriminate between methylated and unmethylated alleles.

We further analyzed the reproducibility of this assay on heterogeneous samples (see Table 1 in the online Data Supplement). We examined the reproducibility by performing 2 independent reactions for each assay. The multiple tests for the 3 loci (Fig. 2C) represent the $CT_{FAM} - CT_{VIC}$ values obtained for these reactions. The δ values of first and second measures were well related in each assay. These results support the QASM assay as capable of yielding reproducible results for complex heterogeneous DNA from clinic samples.

We finally performed a dilution experiment to determine the sensitivity and detection limits of the QASM assay. The CpGenome universal methylated DNA was serially diluted with universal unmethylated DNA. The QASM assay showed that methylation could be detected in the presence of a 10 000-fold excess of unmethylated alleles. We calculated the methylation percentage obtained for each dilution using QASM technique and plotted against the dilution factor (Fig. 2D). The results showed this assay was linear over 4 orders of magnitude in detection of the 3 loci.

COMPARISON WITH BISULFITE PYROSEQUENCING ASSAY

We further compared the quantitative performance of this assay with bisulfite pyrosequencing. We detected the 3 CpG loci in 10 colorectal cancer tissues (see Table 1 in the online Data Supplement). The bisulfite pyrosequencing assays were used to assess the percentage of methylated CpG alleles in the same samples that were run on the QASM assays. These studies were conducted to determine the reliability of the QASM assay. The methylation percentage measured by QASM assays correlated well with that determined by bisulfite pyrosequencing (Fig. 3A; $R^2 = 0.958$ for SGIP1, 0.990 for FHIT, and 0.875 for KAZN; all $P < 0.001$) and also showed a high precision compared with pyrosequencing (Fig. 3B).

COMPARISON WITH ASSAYS NORMALIZED BY INPUT CONTROL REACTION

The ALU-C4-based MethyLight control reaction has been widely used to evaluate input bisulfite-treated DNA concentrations *(18, 19, 23)*. This high-copy control amplicon would be less subject to cancer-associated genetic alterations compared with other single-copy control genes. However, it cannot eliminate the influence of genome-wide copy number variations and mutations on the stability of ALU-C4-based control completely. In the QASM assay, no such input control reaction is needed, and each sample will have an accurate methylation percentage independent from input control. Therefore, we compared the detection performance of these 2 assays in 10 clinical tumor samples. Using bisulfite pyrosequencing as the comparative method, the methylation percentages determined by the QASM assay using the methylated/unmethylated signal ratio were more precise than those determined by MethyLight using the ALU-C4 reaction as input control and full methylated DNA as reference (Fig. 4, A and B). We also found that the SDs of 3 independent measurements in MethyLight were much higher than those in the QASM assay (Fig. 4C). The higher SDs observed in conventional MethyLight may result from stochastic PCR at low template concentrations *(24)*, and the deviations accumulated in 2 independent reactions. However, it is less susceptible in the QASM assay because the signals from the methylated and unmethylated probes were produced with the same primers in the same reaction.

APPLICATION OF QASM ASSAY ON MICROARRAY VALIDATION AND CpG-RICH REGION

We used the assay to technically validate the methylation status of CpGs identified as differentially methylated CpGs on the Illumina MethylationEPIC (EPIC) BeadChip microarray. The EPIC array is the next generation of the HM450 array *(25)*, and 78.2% of 413 745 newly added probes in the EPIC array compared with the HM450 array are located in CpG open seas *(26, 27)*, requiring an assay that can validate single-base methylation in clinical cohorts. Ten human colorectal adenocarcinomas were analyzed (see Table 1 in the online Data Supplement). The QASM assays were used to assess the percentage of methylated CpG alleles at cg05971061 (SGIP1), cg05704547 (FHIT), and cg06887407 (KAZN) in the same samples that were run on the EPIC microarrays. These studies were conducted to determine the reliability of the EPIC microarrays. The relative methylation levels (β values) measured by the EPIC microarray correlated reasonably well with the methylation percentage determined by QASM assays (Fig. 5; $R^2 = 0.987$ for cg05704547, 0.985 for cg05971061, and 0.778 for cg06887407; all $P \leq 0.01$).

Next, we applied the QASM assay to determine single-base methylation in a CGI within the promoter of cysteine dioxygenase type 1 gene (*CDO1*, NM_001801). The primers and methylation allelic and unmethylation allelic probes were designed to avoid CpGs except for the target CpG constrained to being covered by probes (see Fig. 2 in the online Data Supplement). The QASM assay results were linear over serial dilutions of fully methylated DNA and had comparable results with the bisulfite pyrosequencing assay (see Fig. 2 in the online Data Supplement).

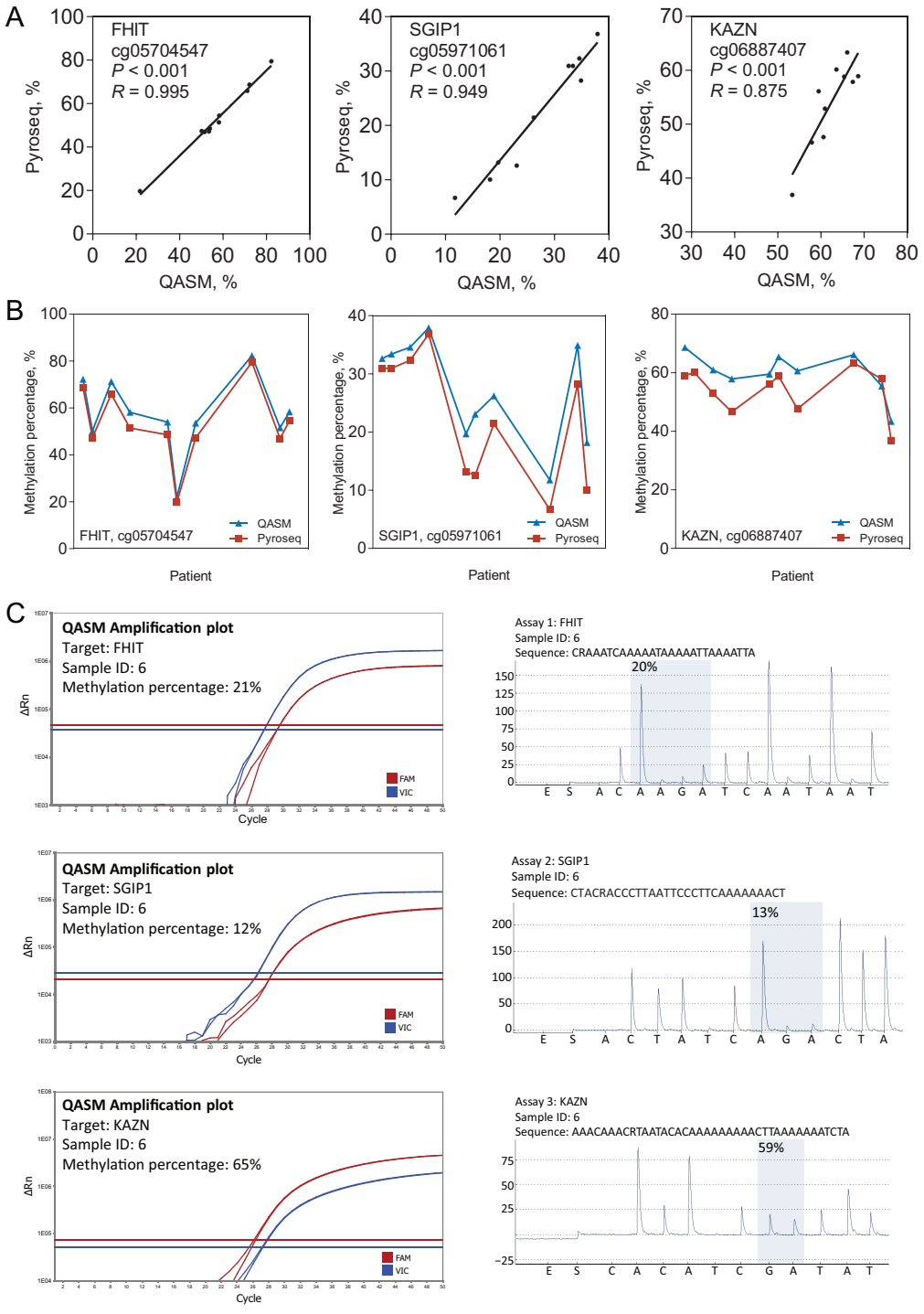

Fig. 3. Comparison of QASM measurements of each CpG site with pyrosequencing measurements in the clinical tumor samples.
The methylation percentages of the target loci in SGIP1, FHIT, and KAZN determined by QASM assay not only correlated well with (A) but also showed the similar value (B) with the methylation percentages determined by pyrosequencing. The representative case of raw methylation data of the 3 loci measured by QASM and pyrosequencing assay is shown in (C). Data represent the mean values of 3 independent experiments.

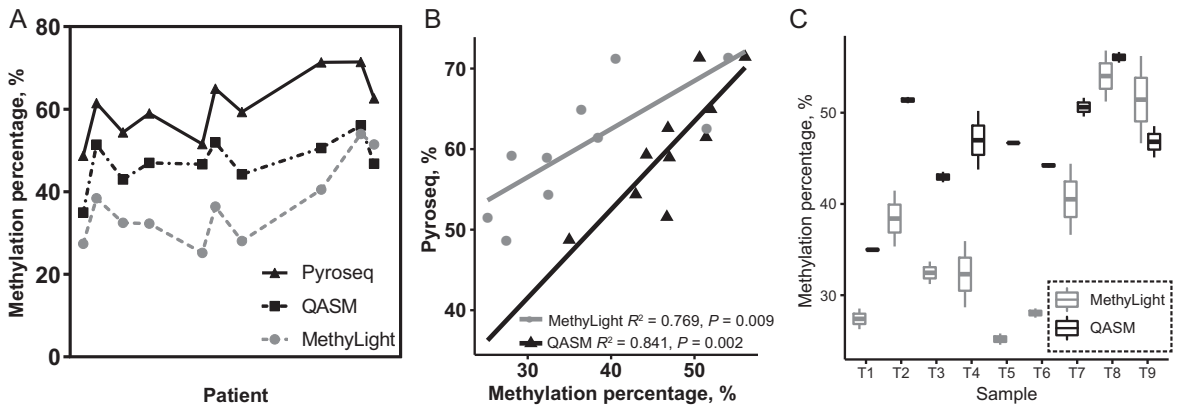

Fig. 4. The calculation system of QASM improved the measure accuracy of conventional MethyLight using input control.

The single CpG in KAZN was measured by QASM assay using methylation / (methylation + unmethylation) × 100. It was also measured by $(\text{methylation / input control})_{\text{test sample}}$ / $(\text{methylation / input control})_{\text{fully methylated reference sample}}$ × 100, which was applied to previous MethyLight techniques. The ALU-C4 reaction was used as an input control. The bisulfite pyrosequencing was used as a reference to compare the accuracy of these 2 calculation systems. (A and B), the QASM assay, without the introduction of input control or reference of fully methylated, is much more precise than previous the MethyLight assay using input control and fully methylated reference. (C), Comparison of mean and SD values of QASM and MethyLight normalized by ALU-C4 reaction. The SDs of 2 independent measurements in conventional MethyLight using ALU-C4 reaction as input control are much higher than in QASM using the methylated/unmethylated signal ratio to eliminate input difference. Data represent the mean values of 3 independent experiments.

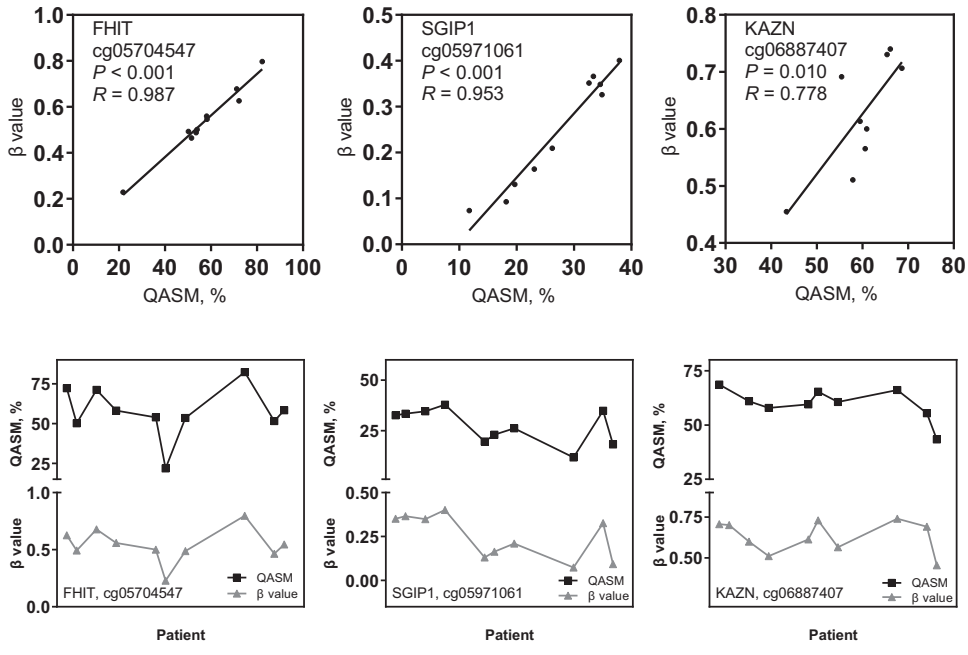

Fig. 5. Use of QASM-based measurements to validate human the Infinium MethylationEPIC microarray screening in tumor samples.

QASM data represent the mean values of 3 independent experiments.

Discussion

We present a PCR-based QASM assay that uses fluorescence-based real-time PCR for the accurate quantification of single CpG methylation. This assay is not only highly analytically specific, sensitive, and reproducible, it is a simplified assay that does not require input control reaction (i.e., ALU-C4) or fully methylated DNA reference. Of note, the quantification of methylation is precise compared with bisulfite pyrosequencing, which is widely accepted to be a most reliable methylation assay.

This QASM technique was designed to determine the methylation of a single CpG dinucleotide and, thus, could yield single-base resolution methylation information if the flanking sequence is eligible to design the primers and paired probes avoiding other CpG dinucleotides with sustained specificity following reduced complexity by bisulfite treatment. This single-base methylation information is obtainable with bisulfite genomic sequencing or bisulfite pyrosequencing previously, which is not cost-effective or feasible for low-throughput validation in large cohorts. This QASM assay showed a high degree of analytical sensitivity, specificity, and reproducibility for single CpG measure. Therefore, this QASM assay can be used to determine the methylation of single CpGs not only in open seas lacking flanking CpGs but also in CGIs or repetitive regions if it is possible to constrain the probes to land over the target CpG with specific amplification (see Fig. 2 in the online Data Supplement).

It is an alternative method to exploit the VeraCode GoldenGate Methylation Assay microarray (Illumina) to validate the single-base methylation of specific loci from the discovery assay, such as the HM450 array *(28, 29)*. Although the VeraCode assay is valuable, it uses the same extension-based principle as the HM450 array and, thus, is limited by its methodology independence of validation from the discovery assay. In addition, it is also limited by the request for a custom-designed bead reader and plate with 96 or 384 specific CpG loci. Therefore, the specific CpG loci discovered from a high-throughput assay could first be validated by the VeraCode assay, and the most promising candidate CpG loci could be further validated by QASM in large cohorts.

The advantages of the QASM assay over existing techniques are higher reproducibility and accuracy. Several aspects of this assay may contribute to these advantages. First, it has a simplified work flow that is not required for an independent input control reaction and, thus, the result is less susceptible to normalization errors caused by cancer-associated aneuploidy and copy number changes. The SDs of 2 independent measurements are much less than those using ALU-C4 reaction as input control (Fig. 4), indicating that the QASM assay avoids the inevitable deviations and errors from the input reaction that will influence the final results. Second, a fully methylated DNA reference is not needed because the methylation percentage is determined by the ratio of methylation / (methylation + unmethylation). It is apparent that the methylation percentage determined by the ratio of target sample to reference DNA is incorrect if the reference DNA is not fully methylated. Finally, its single-base resolution confers independent results that are not influenced by the methylation variants of flanking CpGs. Hence, it is not surprising that the methylation percentage determined by this assay is almost the same as the methylation percentage determined by bisulfite pyrosequencing.

Some limitations of this assay must be noted. The results obtained by QASM could be misinterpreted if the targeted CpG sites or flanking sequence assay oligos bind to is affected by nucleotide polymorphisms or mutations. This is not a limitation specific for this assay but applies to all methylation assays, including the MethyLight, bisulfite pyrosequencing, and methylation microarray *(30)*. In addition, this quantitative PCR-based assay is also limited by the potentially unspecific amplification resulting from reduced genomic complexity in bisulfite treatment *(31, 32)*.

In summary, the QASM assay is highly specific and sensitive to determine methylation at single-base resolution with simplified work flow. Moreover, it has a higher reproducibility and accuracy than the existing MethyLight assay. The calculation method using methylation / (methylation + unmethylation) should theoretically be applicable to any fluorescence-based methylation detection method. The QASM assay is potentially useful for methylation-based panels in diagnostic and prognostic applications.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 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; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.*

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