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Circulating Methylated *SEPT9* DNA in Plasma Is a Biomarker for Colorectal Cancer

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BACKGROUND: The presence of aberrantly methylated *SEPT9* DNA in plasma is highly correlated with the occurrence of colorectal cancer. We report the development of a new *SEPT9* biomarker assay and its validation in case–control studies. The development of such a minimally invasive blood-based test may help to reduce the current gap in screening coverage.

METHODS: A new *SEPT9* DNA methylation assay was developed for plasma. The assay comprised plasma DNA extraction, bisulfite conversion of DNA, purification of bisulfite-converted DNA, quantification of converted DNA by real-time PCR, and measurement of *SEPT9* methylation by real-time PCR. Performance of the *SEPT9* assay was established in a study of 97 cases with verified colorectal cancer and 172 healthy controls as verified by colonoscopy. Performance based on predetermined algorithms was validated in an independent blinded study with 90 cases and 155 controls.

RESULTS: The *SEPT9* assay workflow yielded 1.9 μg/L (CI 1.3–3.0) circulating plasma DNA following bisulfite conversion, a recovery of 45%–50% of genomic DNA, similar to yields in previous studies. The *SEPT9* assay successfully identified 72% of cancers at a specificity of 93% in the training study and 68% of cancers at a specificity of 89% in the testing study.

CONCLUSIONS: Circulating methylated *SEPT9* DNA, as measured in the new ^{m}SEPT9 assay, is a valuable biomarker for minimally invasive detection of colorectal cancer. The new assay is amenable to automation and standardized use in the clinical laboratory.

The 2008 US Multi-Society Task Force Colorectal Cancer Screening Guidelines for average- to increased-risk individuals recommend screening methods that include optical colonoscopy, sigmoidoscopy, fecal occult blood test (FOBT),[7] and fecal DNA markers *(1)*. With the American Cancer Society goal of screening 75% of guideline-eligible people by 2015, considerable effort is being invested to find less invasive alternatives to optical colonoscopy. This is evidenced in a growing body of literature on cancer biomarkers measured in serum, plasma, and stool as potential screening tools *(1–3)*.

One of the primary barriers to achieving the screening objectives is patient compliance with currently available test methods. Thus, although stool-based assays such as the guaiac FOBT have been available for decades and have been effective in reducing morbidity and mortality due to colorectal cancer, their usage remains limited *(1)*. The introduction of a blood-based test for assessing colorectal cancer risk that could be administered as a component of standard preventive care could remove a significant obstacle to screening.

To be effective, informative biomarkers are required that can be readily measured in a standard specimen such as plasma or serum. The observation of increased DNA in cancer patients *(4)* and subsequently the identification of this DNA as tumor derived *(5, 6)* spurred the search for amplifiable tumor DNA markers in patient blood. Aberrantly methylated DNA sequences occur frequently in tumors and were detected in the circulation of cancer patients by PCR *(7, 8)*, representing a rich source for such biomarkers *(9, 10)*. In developing a test for colorectal cancer, we recently reported on identification of 3 novel DNA methylation–based biomarkers, *TMEFF2*, *NGFR*, and *SEPT9* (septin 9), and demonstrated the presence of the *SEPT9*

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[7] Nonstandard abbreviations: FOBT, fecal occult blood test; bis-DNA, bisulfite-treated DNA; DME, diethyleneglycoldimethylether; CFF1, cytosine free fragment 1; FRET, fluorescence resonance energy transfer; LOD, limit of detection; FIT, fecal immunochemical test.

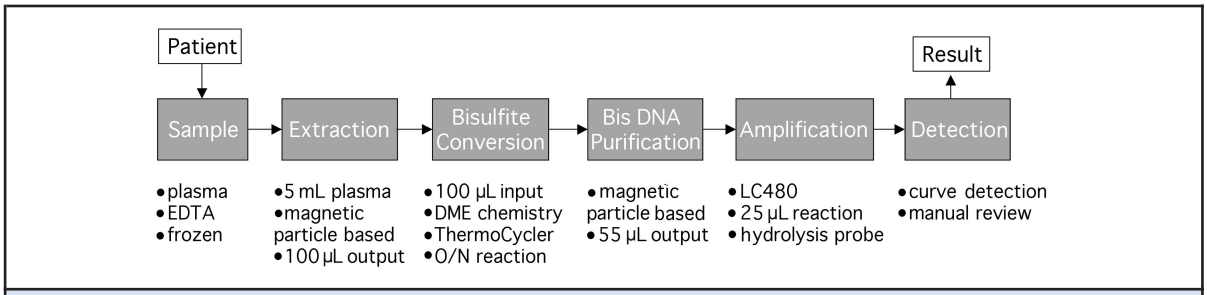

Fig. 1. Outline of the ^{m}SEPT9 assay workflow.

The assay was optimized for an input volume of 4–5 mL plasma. Extraction of DNA from plasma and purification of DNA after bisulfite treatment used magnetic particle methods. Assay results could be reported within 32 h from the start of sample processing. O/N, overnight; LC480, LightCycler 480.

biomarker in the plasma of cancer patients *(11)*. Successful translation of biomarkers to the clinic requires highly sensitive and specific assays, however, since tumor DNA concentration may be <0.1% of the total circulating DNA in blood, particularly in early-stage cancers *(12)*.

We previously developed a research assay to detect hypermethylated *SEPT9* promoter sequences in circulating plasma DNA by PCR amplification of the sequence differences produced by bisulfite treatment of methylated DNA *(13)*. Although alternative methods to measure DNA methylation have been reported *(14)*, our focus has been bisulfite-based technology that allows development of assays at the sensitivity and specificity levels necessary for screening applications. Using the research assay, we correctly identified 72% of the colorectal cancers samples tested at a specificity of 90% in a study of 663 patients *(13)*. The research assay was not easily adaptable to standard laboratory use, however, and its performance was affected by sporadic PCR inhibition.

In this study, we report the development of ^{m}SEPT9, a new assay for the detection of *SEPT9* methylation, and its validation as a colorectal cancer biomarker in 2 independent sample sets. In developing this novel assay, we resolved many technical hurdles, with a focus on enabling routine *SEPT9* methylation analysis in a standard molecular diagnostic laboratory. The development included (1) a new large-volume (5 mL) plasma DNA extraction method to capture fragmented, low-concentration tumor DNA in plasma; (2) a novel bisulfite DNA (bis-DNA) conversion chemistry and particle based bis-DNA purification method to improve recovery of converted DNA and to support an option for carryover prevention *(15)*; and (3) a new real-time methylated *SEPT9* PCR reaction designed to enhance assay sensitivity and eliminate PCR inhibition. These improvements provide a means to translate *SEPT9* and other DNA methylation–based biomarkers from research into the clinic. We report on the performance of the assay, ^{m}SEPT9, in a new, prospectively collected case–control patient set and subsequent verification with a new, independent case–control sample set.

Materials and Methods

STUDY PARTICIPANTS

Ethics review boards at each collection site approved study protocols, and all participants in the study provided written informed consent following local ethics requirements. The study included patients with all stages of colorectal cancer and individuals without diseases of the colon as verified by colonoscopy. The disease status and staging of colorectal cancer patients was obtained from clinical records. All study participants were at least 37 years old, with a majority of patients being 50 and older. Participating subjects did not have a personal history of HIV or herpesvirus B or C infection, cancer other than basal cell skin cancer, or symptoms of severe acute or exacerbated chronic disease.

COLLECTION OF PLASMA

We collected blood samples by phlebotomy using lavender-topped EDTA Vacutainer Tubes (BD Medical Systems) and prepared plasma from blood samples within 4 h of collection by centrifugation of blood tubes (1500*g*, 10 min), transfer to a 15-mL tube, and recentrifugation (1500*g*, 10 min). All plasma from a given patient was pooled and stored at −80 °C.

MEASUREMENT OF *SEPT9*

The ^{m}SEPT9 assay consists of DNA extraction from 4–5 mL plasma, bisulfite conversion of DNA, purification of bis-DNA, and real-time PCR as outlined in Fig. 1 (a detailed protocol is published in Supplemental

Data, which accompanies the online version of this article at www.clinchem.org/content/vol55/issue7). Briefly, extraction of circulating plasma DNA was based on a magnetic particle method, using a modified version of the 4.8-mL Chemagic viral DNA/RNA kit (Chemagen AG). DNA was eluted in 100 μL elution buffer, a 5-μL aliquot of which was used to measure total DNA recovery by real-time PCR. For bisulfite conversion, we added bisulfite salt solutions, organic solvent [diethyleneglycoldimethylether (DME)], and radical scavenger to the eluted DNA in a 0.5 mL elution tube and performed the conversion using a Mastercycler (Eppendorf) for 7 h at 50 °C with 3 thermal spikes at 99 °C. We purified bis-DNA using a magnetic particle based purification kit for bisulfite-converted DNA (Chemagen AG). Purified bis-DNA was eluted in 55 μL elution buffer (10 mmol/L Tris, pH 7.2) and used directly in real-time PCR analysis. The oligonucleotide sequences and assay conditions for the cytosine free fragment 1 (CFF1) (total DNA; CFF1 is a laboratory name for the sequence; see the online Data Supplement for details about this sequence), β-actin (total bis-DNA), and *SEPT9* real-time PCR assays used in this study are provided in online Supplemental Table 1. Real-time PCR analysis was performed on the Lightcycler LC480 (Roche Applied Science) using 96-well reaction plates and the Quantitect Multiplex PCR mastermix (Qiagen).

STUDY DESIGN AND DATA ANALYSIS

We ran the study in batch mode, using positive and negative controls for each extraction and bisulfite batch (online Supplemental Data). Data collection included total genomic DNA recovery following extraction, total bis-DNA recovery, 3 ^{m}SEPT9 PCR measurements on undiluted samples, and 1 ^{m}SEPT9 PCR measurement on a 10-fold diluted sample. Before unmasking the sample identity, we confirmed all PCR results by visual inspection of the PCR curves. Each PCR run included calibrator samples and at least 3 no-template control samples. DNA concentration was determined from calibration curves by linear regression of crossing point values using the second derivative method *(16)*. Samples with <0.001 μg/L bis-DNA (based on the β-actin assay) were excluded from analysis.

For validation of the assay using clinical samples, we followed a training and testing study design. For the training study, we processed and analyzed plasma samples from 100 cancer cases, primarily stage I–III, and 175 noncancer controls. The resulting data were analyzed using multiple algorithms to calculate optimized sensitivity and specificity values. In the test phase of the study, an independent sample set comprising 100 cancer cases and 170 noncancer controls was blinded, processed using the training study workflow, and analyzed using the algorithms agreed to a priori based on the analysis of the training study.

Results

^{m}SEPT9 ASSAY

In this study, we introduced the ^{m}SEPT9 assay, outlined in Fig. 1, which was developed to improve parameters critical for implementation in a standard molecular diagnostics laboratory. The protocol is designed for analysis of plasma collected using standard EDTA collection tubes. During sample preparation, care was taken when transferring plasma to avoid buffy coat cells, and a second centrifugation was added to clear the plasma further before freezing. We and others have found that careful preparation of plasma reduces background DNA resulting from cellular lysis during processing, reducing variability in sample collection *(17)*.

The assay procedure consists of extraction of circulating plasma DNA, bisulfite conversion of the extracted DNA, purification of bis-DNA, and measurement of *SEPT9* methylation status by a real-time PCR assay (Fig. 2). In developing ^{m}SEPT9, our objective was to produce an optimized integrated assay by (1) maximizing plasma input to increase sensitivity; (2) replacing the multiple parallel DNA extractions with a single extraction; (3) replacing purification by ultrafiltration with magnetic particle procedures; (4) reducing the final elution volume to increase DNA concentration; (5) extracting total plasma DNA including high and low molecular weight; (6) reducing the size of the real-time PCR amplicon; (7) overcoming PCR inhibition; (8) providing an approach to prevent carryover; (9) reducing cost; and (10) increasing throughput. We developed assay procedures using surrogate samples that included purified DNA, purified artificially methylated DNA spiked into methylated *SEPT9*-negative plasma, and methylated *SEPT9*-positive plasma spiked into methylated *SEPT9*-negative plasma (online Supplemental Data). The procedures were then validated using clinical samples in case–control studies.

PLASMA DNA EXTRACTION

The extraction procedure, outlined in detail in the online Supplemental Data, was developed for optimal isolation of a broad range of circulating DNA fragment sizes from 4–5 mL of plasma. Several approaches were tested including plasma preconcentration, fluid:fluid extraction, and multiple types of magnetic particles (data not shown), with the optimal method being a single extraction protocol based on the Chemagic viral RNA/DNA protocol (Chemagen AG). The commercially available protocol was modified by Chemagen AG to improve the binding of small fragmented DNA

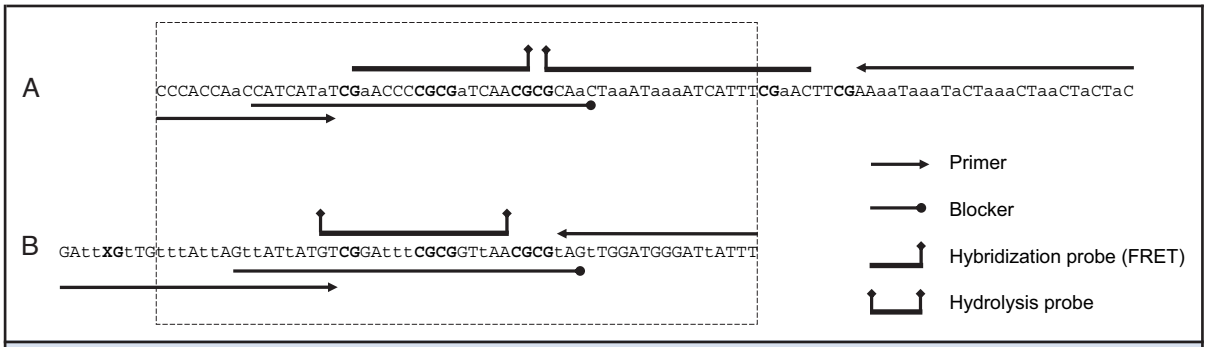

Fig. 2. The ^{m}SEPT9 real-time PCR.

Sequence of the *SEPT9* real-time PCR FRET probe assay (A) compared with the new *SEPT9* hydrolysis probe assay (B). CpG sites are indicated in bold. Lowercase letters a and t indicate positions of bisulfite conversion. The sequence in common between the assays is indicated by the box. Primer binding sites are indicated with arrows, the blocker binding regions are indicated with lines terminating in circles, and the probe binding sites are indicated with lines terminating in diamonds. One primer incorporates a tetrahydrofuran abasic nucleotide indicated in the sequence with an X, and the blocker has a 3′ C3 spacer to prevent extension. The selective amplification of methylated DNA is driven by binding of the blocker to the converted unmethylated sequence at the same CpG positions in both assays. The fluorescently labeled hybridization and hydrolysis probes are methylation specific.

while retaining binding of high molecular weight DNA. We determined optimal binding and washing conditions and established the 100-μL elution volume. The plasma DNA extraction protocol developed with model samples (online Supplemental Data) was confirmed in the training and test set studies, where median DNA concentration for all samples was 5.1 and 3.61 μg/L of input plasma, respectively.

BISULFITE TREATMENT AND PURIFICATION OF bis-DNA

Our objectives for improving the bisulfite procedure were to increase throughput by performing DNA denaturation and conversion in a thermal-cycler and to support automation by magnetic particle–based purification of the bis-DNA. We replaced the organic solvent dioxane with DME, thus reducing the reaction volume from 600 to 320 μL and allowing incubation on a thermal cycler. We tested a variety of magnetic particle systems for purification of bis-DNA (data not shown) and optimized a protocol based on components developed by Chemagen AG. Performance was established with model DNAs (online Supplemental Data) and confirmed in the training and testing studies, where the observed median values (95% CI) were 2.2 μg/L (1.5–3.4) and 1.9 μg/L (1.3–3.0) of input plasma, respectively. We observed an additional benefit, in that the rate of sample dropout decreased to 1.9% in the training set and 2.4% in the test set, a considerable reduction compared with the research assay. An additional benefit of the new bis-DNA purification protocol is that by omission of the desulfonation step, the sulfonated elution product is resistant to UNGase activity, supporting the potential for UNGase-based carryover prevention.

^{m}SEPT9 REAL-TIME PCR

In previous studies, we introduced a real-time PCR assay that uses a blocker oligonucleotide to suppress the amplification of unmethylated target sequences, and fluorescence resonance energy transfer (FRET) MethyLight detection probes *(11, 18)*. In the current study, we developed a modified *SEPT9* real-time PCR assay that produces a 65-nucleotide amplicon, compared with the 91-nucleotide research assay, and uses 5 of the 6 CpG positions interrogated in the original assay (Fig. 2). The specific sequences and reaction conditions are reported in online Supplemental Table 1. Use of a hydrolysis probe allowed us to shorten the probed sequence within the amplicon, and by designing the blocker oligonucleotide to overlap the probe binding site (Fig. 2), we could reduce the amplicon size. We believe the shortened assay improves sensitivity by increased detection of methylated *SEPT9* in fragmented DNA. The analytical performance of the *SEPT9* PCR measured as limit of detection (LOD) for methylated DNA spiked into an unmethylated background was 9.4 pg, representing a relative detection rate of at least 1:5000 (online Supplemental Fig. 1). The new assay was found to have equivalent performance to the research assay in a study using model samples (online Supplemental Fig. 2), so we proceeded to validate the assay with clinical samples in a training and test study.

Table 1. Disease and stage distribution of patient samples in the training and test studies.

	Training set samples				Test set samples			
Sample group	Total	Female	Male	Median age, years (range)	Total	Female	Male	Median age, years (range)
Colorectal cancer	97	33	64	62.5 (37–87)	90	39	51	65 (41–86)
Stage I	22	5	17	64 (47–79)	19	8	11	66 (53–82)
Stage II	38	21	17	63 (37–87)	40	19	21	66 (41–86)
Stage III	34	6	28	60 (40–86)	27	11	16	60 (42–75)
Stage IV	3	1	2	47 (45–62)	4	1	3	66 (53–73)
Control	172	87	85	60 (40–87)	155	91	64	54 (40–90)
Total	269				245			

CLINICAL CASE–CONTROL STUDIES

In the training study, plasma samples were collected from 100 colorectal cancer cases and 175 colonoscopy-verified noncancer controls. Three case and 2 control samples were excluded from data analysis for having <0.001 μg/L bis-DNA (based on the β-actin real-time PCR). A sixth patient was excluded due to a corrected diagnosis of adenomatous polyp, resulting in a final training sample set of 97 cases and 172 controls (Table 1). In the testing study, we collected plasma from 100 cases and 170 controls. Six cases and 13 controls were invalid due to a batch processing error, and 4 cases and 2 controls were excluded for having <0.001 μg/L bis-DNA. Thus the final testing sample set consisted of 90 cases and 155 controls (Table 1). For both training and test studies, we focused collection on stage I–III cancer, but with updated staging information, 3 cases were corrected to stage IV in the training study and 4 cases were corrected to stage IV in the testing study.

In both the training and test studies, we measured total extracted plasma DNA and total bis-DNA with single measurements. PCR was performed in triplicate on undiluted samples. Measurement of a 1:10 dilution of all samples showed no evidence for PCR inhibition with the new assay. Measurement of total DNA showed no significant difference between noncancer controls and stage I–III cancer cases, whereas higher DNA concentrations were observed in some stage IV cancer cases (online Supplemental Fig. 3).

TRAINING STUDY

To optimize the performance of the assay, we analyzed the data with several algorithms as reported in Table 2. For qualitative analyses, a sample was scored as positive or negative by review of the amplification curves. For high sensitivity, samples were considered positive when at least 1 of 3 PCR reactions was positive. As shown in Table 2, the performance (95% CI) for all patients was 75% (65%–83%), whereas for stage I–III sensitivity was 74% (64%–83%), at a specificity of 87% (81%–91%). For high-specificity analysis, samples were counted as positive if at least 2 of 3 curve calls were positive, resulting in an overall sensitivity of 57% (46%–67%), with a sensitivity of 55% (45%–66%) for stage I–III and an improved specificity of 98% (94%–99%).

A third approach to analysis combined quantitative measurement of total DNA and qualitative analysis of the methylated *SEPT9* PCR reactions (Fig. 3A). We observed that the false-positive rate in the high-sensitivity mode increased with increasing total bis-DNA concentration (Fig. 3B) and reasoned that we could establish a total bis-DNA threshold value below which a single positive curve was sufficient for a positive call (high-sensitivity interpretive criteria), and above which at least 2 positive curves were required for a positive call (high-specificity interpretive criteria). We selected the third quartile bound of the β-actin measurement as a threshold DNA concentration (3.4 μg/L), such that 75% of the samples were analyzed according to the high-sensitivity interpretive criteria and 25% were analyzed according to high-specificity criteria. This optimized conditional algorithm resulted in a 73% (63%–82%) sensitivity for stage I–III cancers and 92% (87%–96%) specificity (Table 2). Application of the conditional algorithm resulted in 10 false-positive control samples being properly classified as negative, improving the specificity, and only 1 positive case being reclassified as negative.

TESTING STUDY

The test set comprised 90 valid cancer samples and 155 noncancer controls. The samples were processed in a masked manner, and the results were recorded based on the algorithms established in the training set. The sample key was unmasked on completion of the study, and the results are summarized in Table 2. For stages I–III, we observed a sensitivity of 71% (60%–80%) at a

Table 2. Training and test set results.[a]

	High sensitivity (1/3)		High specificity (2/3)		Conditional qualitative	
Patient Group	Positive/tested	% Positive	Positive/tested	% Positive	Positive/tested	% Positive
Training set results						
Stage I	10/22	45	7/22	32	10/22	45
Stage II	32/38	84	25/38	66	31/38	82
Stage III	28/34	82	20/34	59	27/34	79
Stage IV	3/3	100	3/3	100	3/3	100
Stages I–III	70/94	74	52/94	55	68/94	72
All cancer	73/97	75	55/97	57	71/97	73
Controls	23/172	13 (87)[b]	4/172	2 (98)	12/172	7 (93)
Test set results						
Stage I	10/19	53	5/19	26	9/19	47
Stage II	30/40	75	24/40	60	29/40	73
Stage III	21/27	78	18/27	67	20/27	74
Stage IV	4/4	100	3/4	75	4/4	100
Stages I–III	61/86	71	47/86	55	58/86	67
All cancer	65/90	72	50/90	56	62/90	69
Controls	22/155	14 (86)	7/155	5 (95)	17/155	11 (89)

[a] Shown is the performance of the *m*SEPT9 assay based on different qualitative analyses of triplicate PCR reactions. For the test set, the calling algorithm established in the training set was applied in a blinded fashion to an independent data set.
[b] Specificity for controls in parentheses.

specificity of 86% (79%–91%) in the high-sensitivity analysis, 55% (44%–65%) and 95% (91%–98%) in the high-specificity analysis, and 67% (56%–77%) and 89% (83%–93%) in the conditional qualitative analysis.

Discussion

We have reported that the presence of methylated *SEPT9* promoter DNA in plasma is a candidate biomarker for the detection of colorectal cancer *(11, 13)*. However, there are several hurdles to overcome in translating DNA methylation biomarkers to viable clinical assays. The challenges of marker biology include the low concentration of an early-stage tumor marker in blood and the specificity of a DNA methylation marker in a body fluid that contains DNA from many sources in the body. Technical challenges include handling methods for larger sample volumes, robust chemistry for bisulfite conversion and purification of bis-DNA, and the development of highly sensitive PCR assays for bis-DNA sequences. Finally, challenges in implementation include throughput, automation, and cost. Based on these criteria, the research assay described previously *(13)* would not be viable in a clinical laboratory. We report here on the development and validation of a new assay in which we have eliminated PCR inhibition, simplified and accelerated handling procedures, improved throughput, added automation potential, and reduced costs, while retaining the sensitivity and specificity performance reported in previous studies.

The *m*SEPT9 assay comprises a DNA extraction step, bisulfite conversion of the DNA, purification of bis-DNA, a real-time PCR reaction to measure the DNA methylation status of the *SEPT9* promoter, and an interpretive algorithm to classify samples as positive or negative. Modifications were introduced in each step and tested using surrogate samples produced by spiking methylated *SEPT9* positive plasma into methylated *SEPT9* negative plasma. Subsequently, the new assay was validated using prospectively collected plasma samples from cases and controls. This staged approach allowed us to optimize the assay using readily available materials before testing on valuable clinical specimens.

The primary challenge for the DNA extraction procedure was the plasma volume necessary to achieve high sensitivity. There are few commercially available methods for routine extraction of DNA from 4–5 mL of body fluids, and none that have been optimized for maximal recovery of circulating DNA from plasma. To

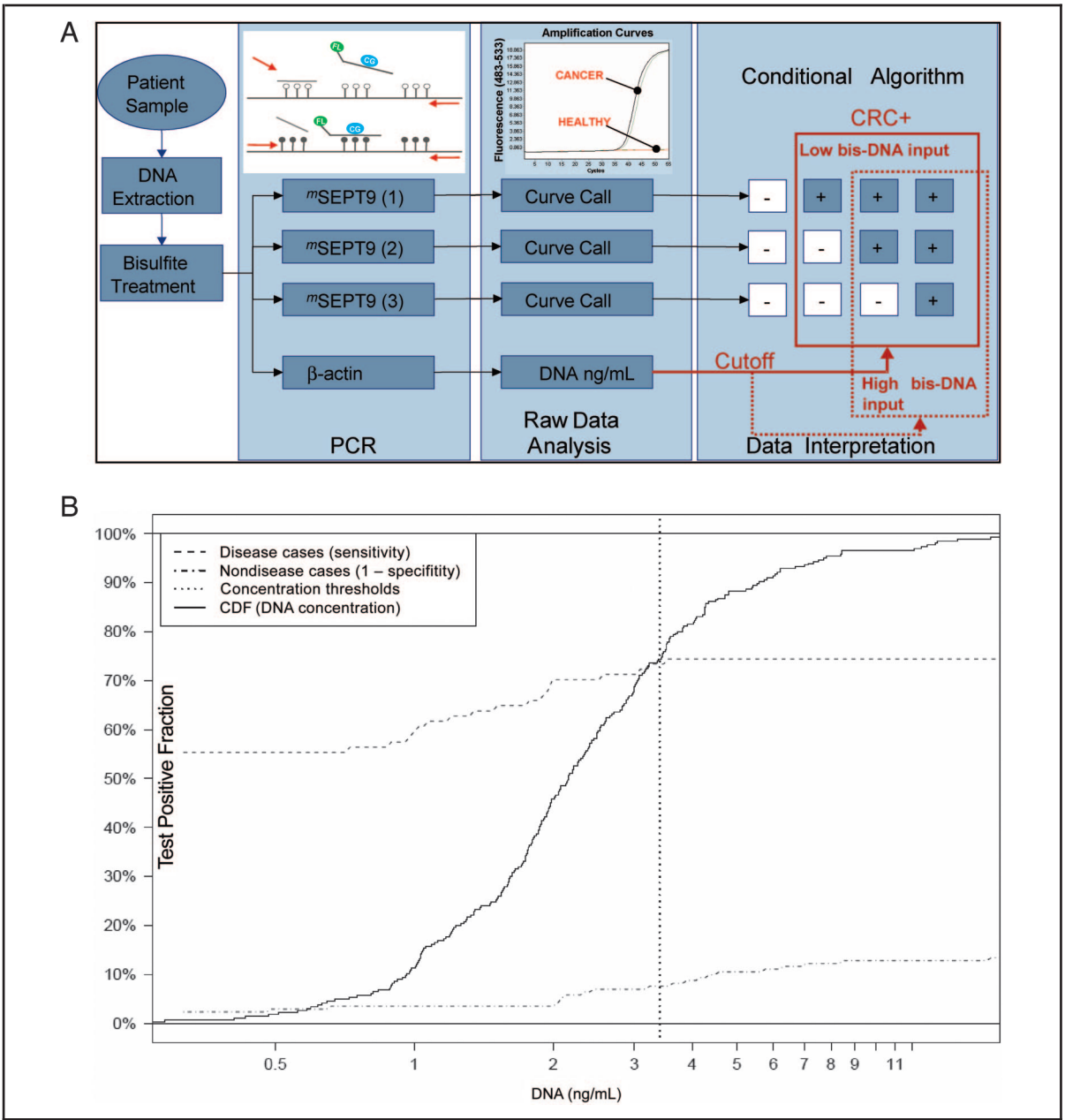

Fig. 3. The ^{m}SEPT9 conditional qualitative algorithm.

(A), Outline of the ^{m}SEPT9 conditional qualitative algorithm. The total DNA concentration after bisulfite treatment is measured for each sample using the β-actin PCR and, based on a cutoff value (3.4 μg/L), samples are categorized for methylated *SEPT9* analysis. Samples with total DNA concentrations below the cutoff are analyzed with the high-sensitivity criteria (at least 1 of 3 calls positive), and samples with DNA concentrations above the cutoff are analyzed with the high-specificity criteria (at least 2 of 3 calls positive). (B), Methylated *SEPT9* detection as a function of total DNA recovery for the training set data. The solid line indicates the cumulative distribution (CDF) of the total DNA concentration in ng/mL on the *x* axis. The dotted vertical line (concentration thresholds) indicates the selected decision point on the total DNA concentration scale (3.4 μg/L). True-positive (dashed line) and false-positive (dashed-dotted line) fractions are displayed as a function of the decision point on the total DNA concentration scale. The performance of the high-specificity rule is represented as the percentage test positive fraction (*y* axis) where the lines cross the left side of the chart, that of the conditional rule is indicated at the vertical line, and that of the high-sensitivity rule where the lines cross the right side of the chart. The decision point was selected to optimize the true-positive fraction while minimizing the false-positive fraction.

process a larger-volume sample *(11*, *13)*, we introduced a single large-volume extraction, improving the ease of handling and reducing costs, while retaining the potential for automation. Optimal results were achieved with the Chemagic 4.8 mL total nucleic acid extraction kit using a modified binding buffer to improve recovery of fragmented DNA. With this extraction protocol, recovery of plasma DNA was in the range observed previously, and we eliminated parallel extractions and pooling steps. Furthermore, the extraction kit is designed for use on a robotic liquid handling platform, providing the potential to automate our protocol.

Similar to the DNA extraction challenge, no bisulfite conversion chemistry is commercially available for the genomic DNA volume produced from a 5-mL plasma DNA extraction. In previous studies, we resolved this issue by pooling and concentrating DNA using microcon filters *(11)* and performed a larger-volume bisulfite treatment with manual heat spikes. In this study, we introduced a new chemistry using the organic solvent DME that allowed a single-tube bisulfite conversion reaction in a thermal cycler. This protocol supported standalone incubation, reducing handling time and increasing throughput compared with the research assay.

After the bisulfite reaction step, the converted DNA is purified and concentrated before PCR. This procedure is required since the bisulfite salts and organic solvents can inhibit the real-time PCR reaction. Commercially available products for bis-DNA purification typically use DNA binding columns or plates, ultrafiltration, or precipitation, which do not meet the volume or automation objectives we set. We successfully replaced the ultrafiltration protocol used in earlier studies with a magnetic particle bis-DNA purification system. Whereas it is frequently reported that the recovery of bis-DNA is <15% *(19*, *20)*, with the β-actin assay we measured a recovery of >50% of DNA following bisulfite treatment. In addition to improved handling and the potential for robotic automation, the bis-DNA produced in this protocol retains the sulfonyl group on the converted residues, conferring resistance to UNGase treatment *(15)*. This potentially enables UNGase-based carryover prevention for bis-DNA, discriminating sulfonyl-uracil residues derived from the conversion reaction of analyte DNA from contaminating uracil-containing amplicons *(15)*. The new purification method for bis-DNA met our development objectives of reduced handling, higher throughput, and potential for automation. In addition, the frequency of sample dropouts was much lower for the new assay compared with the ultrafiltration method in our research assay.

For the real-time PCR step, our objective was to increase throughput by replacing the capillary-based reaction used previously with a standard 96- or 384-well plate assay. In the new *SEPT9* PCR reaction, we modified the fluorescence probe to use hydrolysis chemistry, which can be performed on different real-time platforms. As illustrated in Fig. 2, the new assay measured DNA methylation at the same CpG positions as the previous assay, but was reduced to 65 nucleotides. Similar to the β-actin amplicon, reduction of the *SEPT9* amplicon size has the potential to improve assay sensitivity given the fragmented template DNA produced in a bisulfite treatment. This was also observed for the measurement of mutations in plasma and stool samples from colorectal cancer patients using the BEAMing assay *(3)*. Furthermore, the ^{m}SEPT9 assay showed no evidence of PCR inhibition observed with our research workflow *(13)*.

To validate the ^{m}SEPT9 assay, we assessed its performance in 2 independent, prospectively collected case–control studies. The first study of 97 cases and 172 controls was performed in an open training setting to develop the interpretive algorithms. The second study of 90 cases and 155 controls was performed in a blinded setting to test the algorithms selected in the training study. For the analysis of clinical specimens, our interpretive algorithm was based on the qualitative analysis of 3 replicates of the *SEPT9* PCR and the quantitative estimate of total bis-DNA (β-actin PCR). Analysis of total DNA recovery revealed no differences in concentration between noncancer controls and stage I–III cancer cases. In some patients, particularly those with late-stage cancers, we observed an increase in the concentration of total DNA in the blood, in rare cases reaching μg/mL quantities. These observations corroborate reported observations that total circulating DNA concentration is an indicator for metastatic cancer *(21)*, as is further demonstrated in a recent article using BEAMing technology *(22)*. However, we have demonstrated that for early stage colorectal cancer (I–III), specific biomarkers are essential for cancer detection, and that in contradiction with some reports *(23)*, total plasma DNA concentration is not a useful biomarker (online Supplemental Fig. 3).

For valid samples (>0.001 μg/L bis-DNA), the ^{m}SEPT9 results could be calibrated to maximize sensitivity (a single positive replicate is scored positive) or specificity (2 or 3 replicates are required to be positive for a positive call). In the training study, we observed a sensitivity of 74% at a specificity of 87% using the high-sensitivity criteria and a sensitivity of 55% at a specificity of 98% using the high-specificity criteria. Based on the observed correlation of false-positive results with increased DNA concentration, we applied a DNA concentration threshold to assign samples to either high-

specificity or high-sensitivity interpretive criteria. Using this conditional qualitative analysis, we observed a sensitivity of 73% at a specificity of 92% in the training study. Applying this algorithm in the blinded testing study, we observed a sensitivity of 67% at a specificity of 89%. The results for both the training and test studies corroborate our previous findings, in which we reported that methylation of the promoter region of *SEPT9* was highly correlated with the presence of colorectal cancer *(11*, *13)*. The combined results of these multiple studies strongly support the potential for methylated *SEPT9* as a biomarker for colorectal cancer.

Although hypermethylation of *SEPT9* promoter regions has been reported in head and neck *(24)* and ovarian *(25)* cancer, it is difficult to compare these reports directly with our results for colorectal cancer because they measure unknown or different regions of the *SEPT9* promoter and are reported for cell lines and tissues, in comparison with our results for plasma. In previous studies, we measured *SEPT9* methylation in other cancers with a survey of plasma for 96 patients representing 7 major cancers, and observed 11 of 96 positive patients, or a specificity of 89% *(13)*, similar to the specificity in our current control group.

The performance of the ^{m}SEPT9 assay described above compares favorably with other minimally invasive tests reported in the literature. Results for guaiac FOBT are well summarized in the 2008 screening guidelines *(1)*, falling into low-sensitivity (approximately 35%) high-specificity (95%) tests or higher-sensitivity (65%) lower-specificity (86%) tests. Our performance is similar to immunochemical FOBT [fecal immunochemical test (FIT)] in which, for example, Morikawa et al. *(26)* reported 66% sensitivity at a specificity of 95% in a study of >20 000 patients using FIT, whereas Allison et al. *(27)* reported 81% sensitivity (with only 11 cancers) at a 95% specificity in a >5000 patient study. The ^{m}SEPT9 test also compares well with results for stool DNA testing, for which sensitivity of 58% and specificity of 85% was reported in a recent study *(28)*. Considering the compliance issues associated with stool-based screening, we believe that the plasma based ^{m}SEPT9 test can fulfill a significant unmet need. Thus, a prospective collection of plasma samples from subjects in the colorectal cancer screening guideline-eligible population is currently underway to evaluate the clinical performance of ^{m}SEPT9 for the detection of invasive colorectal adenocarcinoma (PRESEPT study, clinicaltrials.gov, identifier NCT00855348).

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