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Enhanced Performance of DNA Methylation Markers by Simultaneous Measurement of Sense and Antisense DNA Strands after Cytosine Conversion

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BACKGROUND: Most existing DNA methylation-based methods for detection of circulating tumor DNA (ctDNA) are based on conversion of unmethylated cytosines to uracil. After conversion, the 2 DNA strands are no longer complementary; therefore, targeting only 1 DNA strand merely utilizes half of the available input DNA. We investigated whether the sensitivity of methylation-based ctDNA detection strategies could be increased by targeting both DNA strands after bisulfite conversion.

METHODS: Dual-strand digital PCR assays were designed for the 3 colorectal cancer (CRC)–specific methylation markers *KCNQ5*, *C9orf50*, and *CLIP4* and compared with previously reported single-strand assays. Performance was tested in tumor and leukocyte DNA, and the ability to detect ctDNA was investigated in plasma from 43 patients with CRC stages I to IV and 42 colonoscopy-confirmed healthy controls.

RESULTS: Dual-strand assays quantified close to 100% of methylated control DNA input, whereas single-strand assays quantified approximately 50%. Furthermore, dual-strand assays showed a 2-fold increase in the number of methylated DNA copies detected when applied to DNA purified from tumor tissue and plasma from CRC patients. When the results of the 3 DNA methylation markers were combined into a ctDNA detection test and applied to plasma, the dual-strand assay format detected 86% of the cancers compared with 74% for the single-strand assay format. The specificity was 100% for both the dual- and single-strand test formats.

CONCLUSION: Dual-strand assays enabled more sensitive detection of methylated ctDNA than single-strand assays.

Introduction

Detection of circulating tumor DNA (ctDNA) in the blood provides a minimally invasive diagnostic avenue for patients with cancer. Many cancer-specific markers have been used to detect ctDNA, including somatic mutations *(1–5)*, DNA fragmentation patterns *(6, 7)*, and DNA methylation patterns *(8–10)*. In particular, DNA methylation markers appear promising for early detection of cancer because aberrant DNA methylation occurs early in tumor development and often affects the same genomic regions among patients *(11, 12)*. Most existing DNA methylation-based methods for detection of ctDNA are based on conversion of unmethylated cytosines to uracils by either enzymatic or bisulfite treatment. After conversion, the 2 DNA strands are no longer complementary (Fig. 1, A). Consequently, targeting only 1 DNA strand, which is the most commonly used approach for DNA methylation detection, utilizes only half of the available DNA for cancer detection (Fig. 1, B). We recently developed a colorectal cancer (CRC)–specific ctDNA detection test based on 3 DNA methylation markers (the TriMeth test) *(13)*. The test comprises 3 methylation-specific droplet digital PCR (ddPCR) assays targeting the DNA sense strands of genomic regions in the first exon of *C9orf50*, *KCNQ5*, and *CLIP4*. The TriMeth test has sensitivity of 85% and specificity of 99% in plasma from patients with stage I to IV CRC and healthy controls *(13)*. Potentially, TriMeth sensitivity could be increased if the DNA antisense strands were also targeted. Because the template DNA is single-stranded after cytosine conversion, the 2 strands of the DNA fragments from targeted regions end up in different droplets during ddPCR droplet formation. Consequently, targeting both strands

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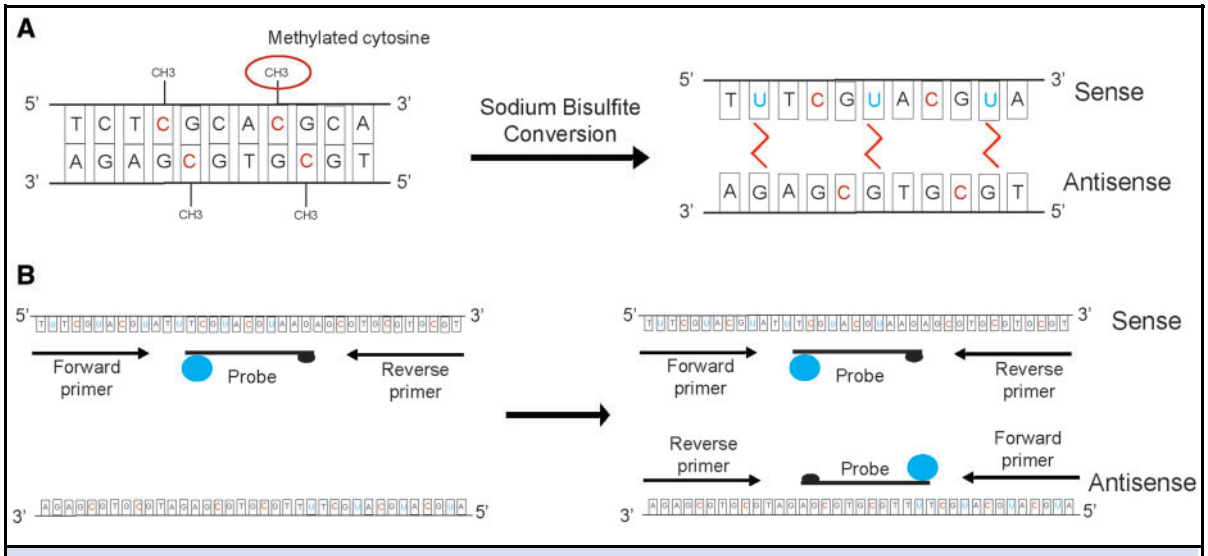

Fig. 1. Study strategy. A, During sodium bisulfite conversion of DNA, unmethylated cytosines are deaminated to uracils resulting in noncomplementary sense and antisense DNA strands. Therefore, methylation-specific ddPCR assays targeting only the bisulfite-converted sense DNA strand will not amplify the antisense strand. B, The study strategy was to develop methylation-specific ddPCR assays targeting the bisulfite-converted antisense DNA strand of the 3 DNA methylation markers *C9orf50*, *KCNQ5*, and *CLIP4* and to use these in duplex reactions combined with previously published assays targeting the sense strand of these markers.

would theoretically double the number of methylated DNA copies detected by ddPCR in plasma from CRC patients. With this study, we aimed to investigate whether the sensitivity of the TriMeth test could be increased by applying methylation-specific ddPCR assays targeting both the sense and the antisense strands of the bisulfite-converted DNA.

Materials and Methods

PATIENT SAMPLES AND CHARACTERISTICS

Informed consent was obtained from all participants, and the use of their biological material and clinical data was approved by the National Committee on Health Research Ethics and by the Danish National Data protection agency. Tumor samples from CRC patients were collected at the Surgical Departments of Aarhus University Hospital, Randers Regional Hospital, and Herning Hospital in Denmark. The tissue was snap-frozen in liquid nitrogen within 30 minutes from tumor resection and stored at −80 °C. Peripheral blood leukocytes (PBLs) were isolated from 10 mL of blood collected from presumed healthy Danish blood donors aged 50 years or older at the blood bank of Aarhus University Hospital. Preoperative plasma samples were collected from patients diagnosed with stage I to IV CRC at the Surgical Departments of Aarhus University Hospital, Randers Regional Hospital, and Herning Hospital. Control participants with a colonoscopy-verified clean colon, no previous cancer diagnosis, and no comorbidities except for hypertension were randomly selected among fecal immunochemical test-positive individuals in the Endoscopy III trial, which enrolled participants from the Danish national CRC screening program from 2014 to 2016 *(14)*; these individuals composed a relevant "average risk" control group with the appropriate age range for assessment of performance of the specificity of a test like the TriMeth. Blood collection and plasma isolation from patients and controls were performed using the same standard operating procedure. Patient characteristics and demographic information are shown in Table 1 and online Supplemental Table 1.

ETHICAL ASPECTS

The prospective collection of plasma samples from participants in the Danish national CRC screening program was approved by the Scientific Ethics Committee of the Capital Region Denmark (j. no. H-4-2013-050) and the Danish Data protection Agency (j. nos. HVH-2013-022/2007-58-0015). Use of the existing plasma and tissue collections was approved by the Committee on Health Research Ethics (j. nos. 1999/4678 and H-3-2009-110) and the Danish Data Protection Agency (j. nos. 2007-58-0010 and 2008-41-2252).

Table 1. Patient characteristics for the plasma cohort.

	Plasma samples	
	CRC	Controls
Total, n	43	21
Sex, n (%)		
Female	19 (44)	9 (43)
Male	24 (56)	12 (57)
Age, years		
Median (interquartile range)	69 (66–79)	70 (65–75)
UICC[a] stage, n (%)		
Stage I	15 (35)	...
Stage II	16 (37)	...
Stage III	6 (14)	...
Stage IV	6 (14)	...
Tumor size, mm		
Mean (SD)	40.7 (17.2)	...
Histological type, n (%)		
Adenocarcinoma	41 (95)	...
Mucinous adenocarcinoma	2 (5)	...
Localization, n (%)		
Right (cecum, ascending, transverse)	20 (47)	...
Left (descending, sigmoid)	7 (16)	...
Rectum	16 (37)	...

[a]UICC, Union of International Cancer Control.

DUAL-STRAND ASSAY DESIGN

In this study, 4–7 assays with primers and probes exclusively specific for methylated cytosine-converted DNA were designed for each marker (a total of 17 assays). As was done for the sense assays, locked nucleic acids were incorporated into primers and probes to increase assay specificity and to reduce amplicon lengths *(13, 15)*. To ensure that the majority of circulating cell-free DNA (cfDNA) templates (median size of 167 bp) contained a full-length amplicon, amplicon lengths were kept below 80 bp *(16)*. The antisense probes were labeled with the same fluorophore as the corresponding sense assay probe, resulting in only 1 fluorescent signal from each DNA methylation marker. Assay details, including primer and probe sequences, are shown in online Supplemental Table 2.

TISSUE AND BLOOD PROCESSING AND DNA ISOLATION

DNA was extracted from fresh frozen tissue using the Gentra Puregene Tissue Kit (Qiagen), as specified by manufacturer. DNA from PBLs was purified on a QIAsymphony robot (Qiagen) using the QiaSymphony DSP DNA mini kit (Qiagen), as specified by manufacturer. The purified DNA was eluted in a final volume of 100 μL in 1.5-mL Eppendorf tubes (Eppendorf AG) and stored at −80 °C until use. Whole blood was collected in BD Vacutainer K2 EDTA tubes (Becton Dickinson) and processed within 2 hours from venipuncture. Plasma was separated from cellular components by double centrifugation at 3000*g* for 10 minutes at 20 °C and stored in cryotubes (Techno Plastic Products AG) at −80°C until the time of DNA extraction. Plasma was thawed at room temperature, and cfDNA from 16 mL of plasma was extracted using a QIAsymphony robot and the QIAamp® Circulating Nucleic Acids kit (Qiagen; protocol CF2000_CR2598_ID506_V1), as specified by manufacturer. The purified cfDNA was eluted in a total volume of 120 μL in LoBind tubes (Eppendorf) and stored at 4°C until use (<4 days). DNA purification efficiency and contamination by lymphocyte DNA was estimated for quality control of the plasma DNA, as described previously *(4, 13, 17)*. Briefly, fragments of soybean CPP1 DNA was combined with each plasma sample before purification. The purification efficiency was estimated from the percentage of recovered CPP1 fragments after cfDNA purification using an assay targeting the CPP1 fragments. Contamination with lymphocyte DNA was calculated using an assay (the Peripheral Blood Leukocyte [PBC] assay) targeting the Immunoglobulin Heavy (IGH) chain rearrangement that is specific for B lymphocytes. Average purification efficiency was 72% (95% CI, 68.6–74.8). No samples showed contamination with lymphocyte DNA.

SODIUM BISULFITE TREATMENT

Before bisulfite treatment, cfDNA was vacuum dried (speedVac, Concentrator plus 5350; Eppendorf AG) at 30 °C and resuspended in 20 μL AccuGENE™ Molecular Biology Water (Lonza). The EZ-96 DNA Methylation-Direct™ MagPrep kit (Zymo Research) was used for bisulfite conversion of all DNA samples, according to the manufacturer's instructions, using 60 μL CT conversion reagent, 280 μL M-Binding Buffer, 5 μL MagBinding Beads, 185 μL M-Wash Buffer, 93 μL M-Desulphonation Buffer, and 25 μL M-Elution Buffer. As positive and negative controls, fully methylated and unmethylated control DNA was included for all reactions. Bisulfite-converted cfDNA was analyzed using ddPCR immediately after bisulfite conversion. Other bisulfite-converted DNA samples were stored at −20 °C until use (<1 week).

DNA QUANTIFICATION BEFORE AND AFTER BISULFITE CONVERSION

The ddPCR was applied for DNA quantification using assays amplifying 2 reference target regions on chromosome 1 (CF assay) and chromosome 3 (Chr3 assay), as described previously *(13)*. Primer and probe sequences can be found in online Supplemental Table 2. The CF assay was designed to amplify a cytosine-free genomic region unaffected by bisulfite treatment. Thus, the CF assay was used for DNA quantification and recovery assessments after bisulfite conversion. Recovery was calculated as the quantity after bisulfite conversion divided by the quantity before.

DROPLET DIGITAL PCR

All ddPCR experiments were conducted on the Droplet Digital PCR System (Bio-Rad) according to the Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE) guidelines (online Supplemental Table 3) and manufacturer's instructions (Bio-Rad). The reaction mastermix included 2–9 μL template DNA, 18 pmol forward primer, 18 pmol reverse primer, 5 pmol probe, 2xSupermix for Probes (no UTP; Bio-Rad), and AccuGENE™ Molecular Biology Water (Lonza) to a final volume of 22 μL. A list of the ddPCR assays used is provided in online Supplemental Table 2. Droplets of 1 nL were generated on the QX200 AutoDG Droplet Generator (Bio-Rad). The mean number of accepted droplets per well was >15 000 for native DNA and >14 000 for bisulfite converted DNA. After droplet generation, the samples were amplified by PCR on a S1000 Thermal cycler (Bio-Rad) using the following program: 95 °C for 10 minutes, 45 cycles of 95 °C for 30 seconds and 56 °C for 1 minute, and 1 final cycle of 98 °C for 10 minutes. PCR products were stored at 4 °C for up to 18 hours before analysis on a QX200 reader (Bio-Rad). Positive and no-template controls were included for each assay, and for methylation-specific assays, a negative control was also included. For the methylation-specific assays, the positive control was 5 ng methylated DNA and the negative control was 66 ng nonmethylated DNA (Zymo Research). For the CF, Chr3, and PBC assays, the positive control was 5 ng leukocyte DNA. For the CPP1 assay targeting the soybean CPP1 fragments, the positive control was 7000 copies of CPP1 fragments. For tumor tissue and PBL samples, the DNA input was 5 ng and 66 ng, respectively (quantified before bisulfite conversion). Quantasoft v1.7 software (Bio-Rad) was used to analyze the data.

Results

TRIMETH DNA METHYLATION MARKERS

The previously reported TriMeth test for detection of CRC-specific ctDNA targets the cytosine converted DNA sense strand of the 3 DNA methylation markers *C9orf50*, *KCNQ5*, and *CLIP4 (13)*. These markers were selected based on a comprehensive bioinformatic analysis showing that they were highly CRC specific and not methylated in other common cancers or PBLs from healthy individuals *(13)*. Because most cfDNA in healthy individuals originates from PBLs *(18)*, an increase in the methylation level of the *C9orf50*, *KCNQ5*, and *CLIP4* markers with age might lead to false positives in older healthy controls. Therefore, we investigated whether the methylation levels of the 3 markers were affected by age in PBLs from healthy individuals and individuals with inflammatory diseases (ulcerative colitis, Crohn disease, and arthritis). From this, we found that the methylation levels of the markers were independent of age and inflammatory disease (online Supplemental Figs. 1–3). To investigate whether targeting both the sense and antisense strands would increase the sensitivity of the TriMeth test, we designed antisense assays targeting the same genomic region as the previously designed sense assays. In total, 17 antisense assays (7 assays targeting *C9orf50*, 4 assays targeting *KCNQ5*, and 6 assays targeting *CLIP4*) were designed. An overview of the study workflow is shown in Fig. 2 (for a detailed description of assay design, see Materials and Methods).

INITIAL ASSAY TESTING

Initially, we investigated whether the 17 antisense assays produced a single PCR product. The assays were applied to bisulfite-converted methylated control DNA, and the resulting PCR products were visualized by agarose gel electrophoresis (data not shown). Five assays were eliminated from further analysis given nonspecific DNA amplification. The remaining 12 assays were evaluated by ddPCR using methylated control DNA as a template. Four assays showed poor separation of negative and positive droplets and were excluded. The specificity toward methylated DNA was assessed by applying the remaining 8 assays to samples with 20 000 copies of unmethylated control DNA. None of the assays amplified unmethylated DNA templates. The 8 antisense assays were duplexed with their corresponding sense assays and applied to methylated control DNA. Two of the 8 dual-strand assays showed poor separation of positive and negative droplets and were excluded. Linearity and sensitivity of the remaining 6 dual-strand assays (2 assays for each marker) were investigated using a 6-point dilution series of 10, 30, 50, 110, 260, and 540 copies of methylated DNA mixed with 20 000 copies of unmethylated DNA. Three assays passed the linear amplification test (r^2 = 0.9815–0.9993). These were all able to detect 10 copies of methylated DNA in a background of 20 000 copies of unmethylated DNA (online Supplemental Fig. 4). As expected, the dual-strand

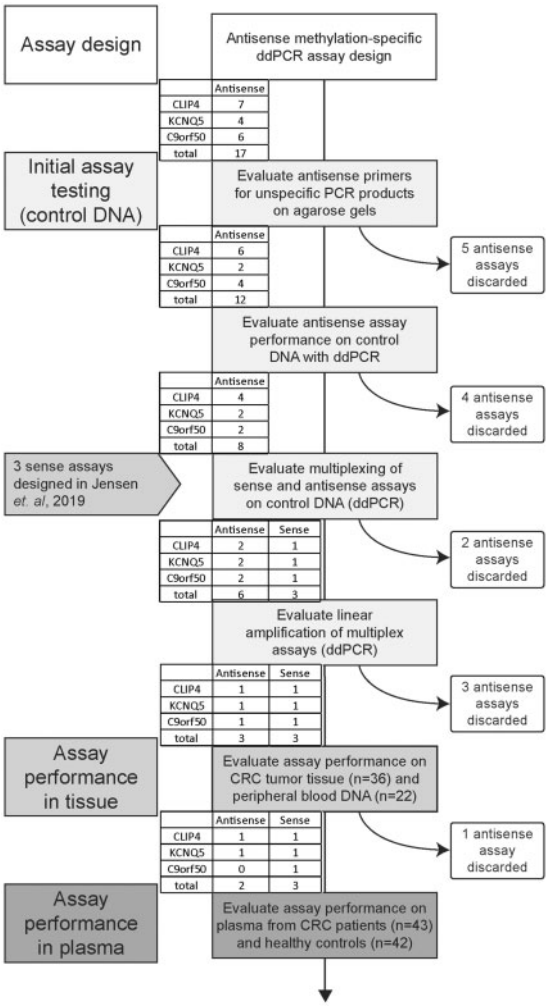

Fig. 2. Study workflow. Seventeen antisense methylation-specific ddPCR assays were designed for the *C9orf50*, *KCNQ5*, and *CLIP4* DNA methylation markers (4–7 assays per marker). Assays were excluded if they produced unspecific PCR products, as visualized by agarose gel electrophoresis. Assays with poor separation of positive and negative droplets in ddPCR experiments using methylated control DNA as a template were eliminated. Assays were tested in duplex with their corresponding sense assays and excluded if they did not amplify methylated control DNA or failed to efficiently separate positive and negative droplets in ddPCR experiments. Dual-strand assay performance was evaluated on colorectal tumor tissues (n = 36) and peripheral blood leukocyte DNA (n = 22). The *C9orf50* dual-strand assay showed positive signals in some PBLs and thus was excluded from further analyses. The *KCNQ5* and *CLIP4* dual-strand assays were evaluated in plasma from CRC patients (n = 43) and healthy controls (n = 42) and compared with the performance of the sense single-strand assays in the same plasma samples.

assays quantified almost 100% (average slope, 0.9188; range, 0.8489–1.0414) of the methylated DNA input, whereas sense assays quantified approximately 50% (average slope, 0.5809; range, 0.4267–0.6703) (online Supplemental Fig. 4).

ASSAY PERFORMANCE IN CRC TUMOR TISSUE AND BLOOD

In the original format of the TriMeth test, *KCNQ5* and *C9orf50* sense assays were applied as a duplex, and the *CLIP4* sense assay was applied as a duplex with the CF control assay. For testing of the novel dual-strand versions of the 3 markers, *KCNQ5* and *CLIP4* were again applied in combination with *C9orf50* and CF, respectively. The performance was compared with the performance of the sense single-strand assays on DNA from 36 early stage colorectal tumors. All assays, dual- and single-strand, detected methylated DNA in all tumors (Fig. 3). The detected number of methylated DNA copies was approximately 2-fold higher for dual-strand assays compared with single-strand assays (*KCNQ5*, 1.96; *CLIP4*:, 1.95; *C9orf50*, 2.24) (Fig. 3, D, E, and F). To evaluate the clinical specificity, the same assays were tested in 22 PBL DNAs (20,000 DNA copies input per ddPCR reaction). The dual-strand assays for *KCNQ5* and *CLIP4* showed almost no signal in the PBL samples (Fig. 3, A and B). The *C9orf50* dual-strand assay showed a weak signal in a higher fraction of the PBLs than the sense assay (Fig. 3, C). Because an increase in the number of *C9orf50* false-positives might decrease the specificity of the TriMeth test, the *C9orf50* antisense assay was excluded from further analysis.

ASSAY PERFORMANCE IN PLASMA

The performance of the dual-strand assays was compared with the performance of the sense single-strand assays in plasma from 43 patients with stage I to IV CRC and 21 colonoscopy-confirmed, age-matched controls (Table 1). For a direct comparison, the dual-strand assays and the sense single-strand assays were tested on the same plasma samples (equivalent cfDNA inputs for all reactions). Bisulfite-converted cfDNA from 4 mL of plasma was used as the input for each ddPCR reaction (median cfDNA input after bisulfite conversion: cases, 3320 genome equivalents (range, 1180–23 520); controls, 2850 genome equivalents (range, 1026–7060). The mean bisulfite-conversion recovery was 43.1% (95% CI, 40.4–45.8). The *CLIP4* and *C9orf50* single- and dual-strand assays and the KCNQ5 dual-strand assay detected no methylated DNA in the plasma of controls (Fig. 4, A, B, and C). The *KCNQ5* single strand assay detected methylated DNA in 1 of the 21 controls (5%) but only 1.4 copies in the 4 mL of plasma.

By contrast, this assay detected an average of 16.2 copies in 32 of 43 (74%) of the plasma samples from

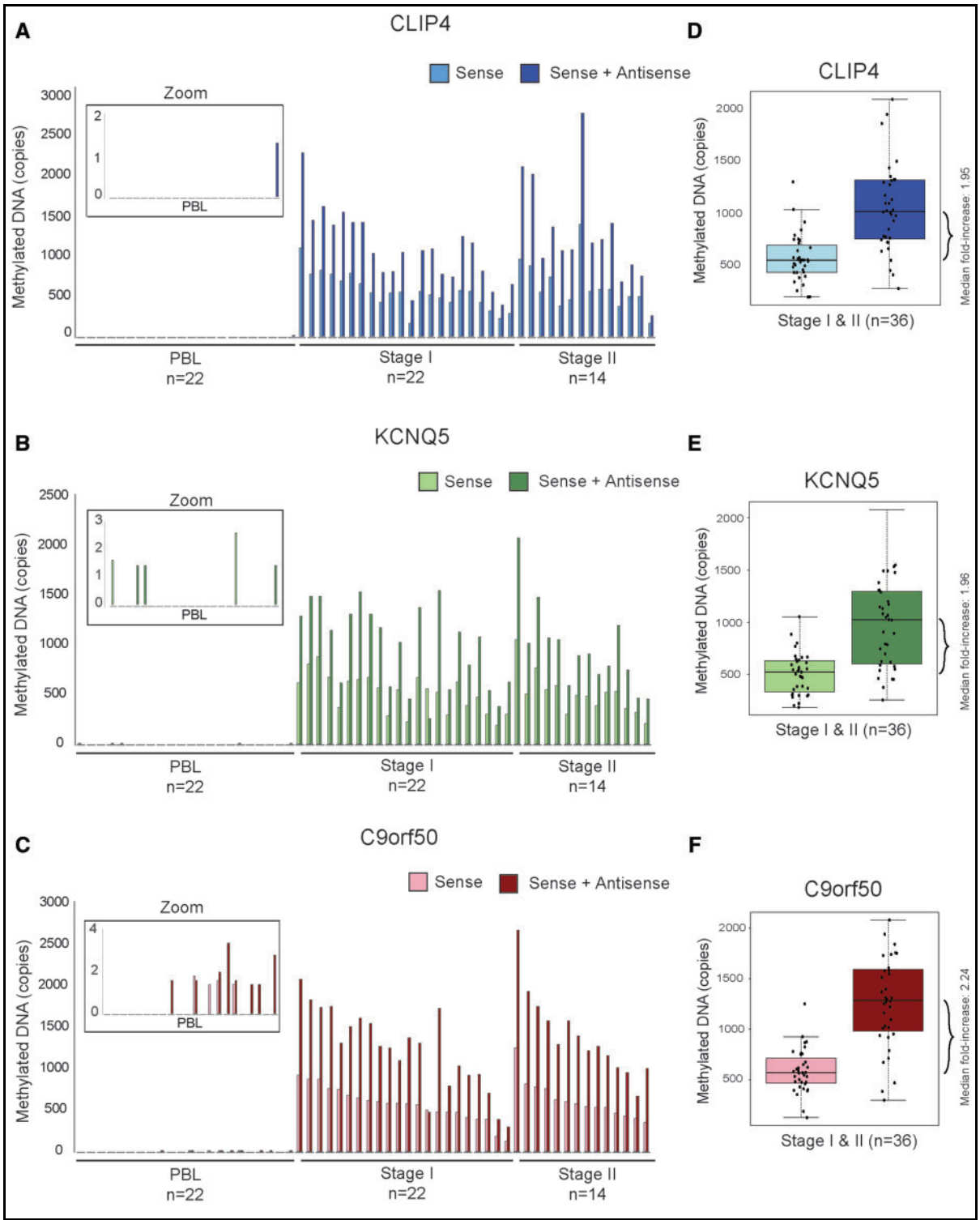

Fig. 3. Performance of methylation-specific single- and dual-strand assays on PBL DNA and colorectal tumor tissues. Methylated DNA copies detected by sense and antisense dual-strand assays and sense single-strand assays in PBL and colorectal tumor tissues (stages I and II) are shown for *CLIP4* (A), *KCNQ5* (B), and *C9orf50* (C). Box plots show the signal increase in colorectal tumor tissue for *CLIP4* (D), *KCNQ5* (E), and *C9orf50* (F).

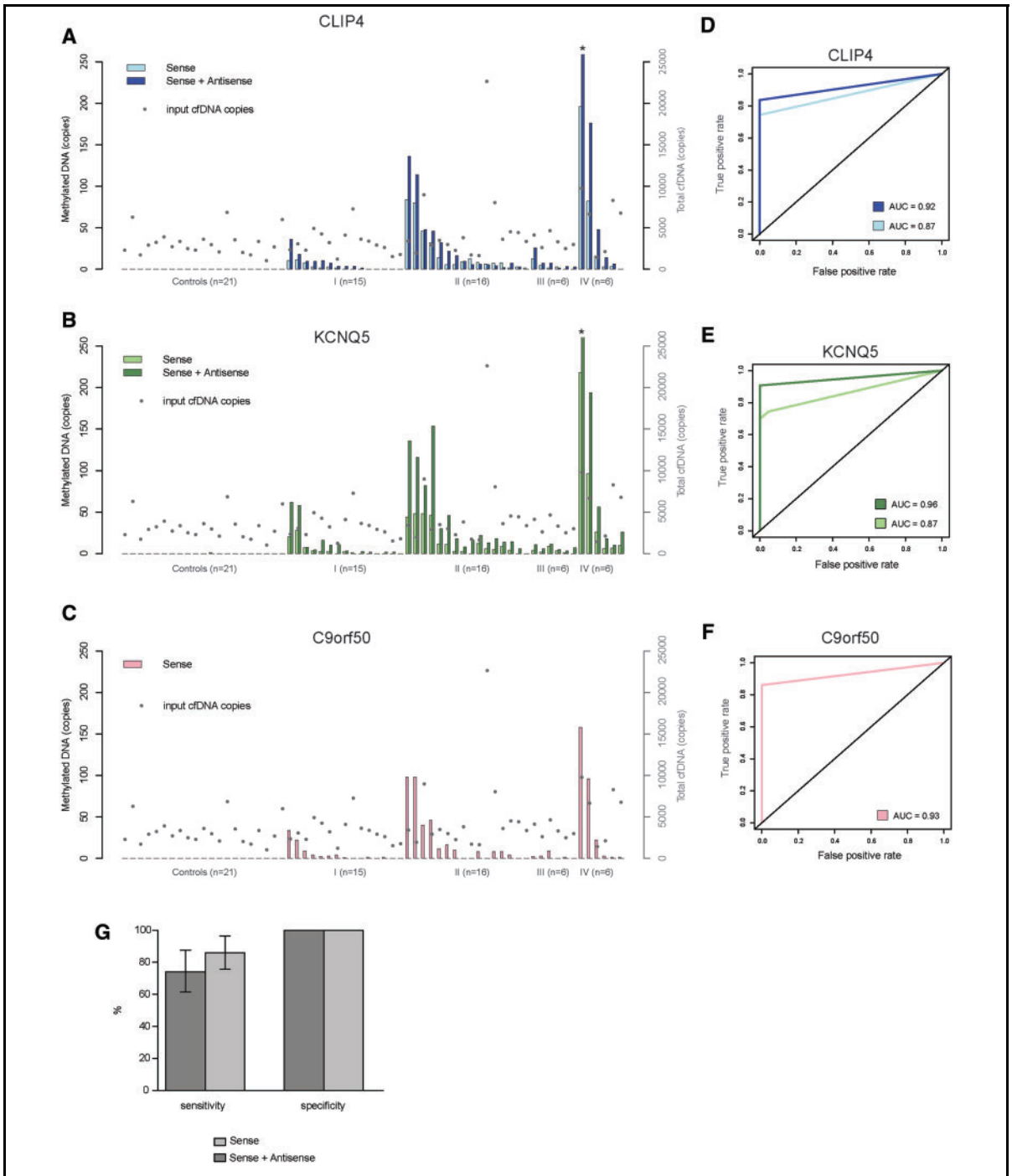

Fig. 4. Performance of methylation-specific single- and dual-strand assays on plasma from CRC patients and controls. Methylated DNA copies detected by single- and dual-strand assays in plasma from patients with stage I and II CRC (n = 43) and age-matched controls (n = 21) are shown for *CLIP4* (A), *KCNQ5* (B), and *C9orf50* (C). Equal amounts of cfDNA input were used for single- and dual-strand assay reactions. The cfDNA quantities are illustrated as gray dots. Asterisks indicate that >250 ctDNA copies were detected (452 methylated copies of DNA for *KCNQ5*, 360 methylated copies of DNA for *CLIP4*). ROC curves and AUCs based on ctDNA copies detected in plasma are shown for single- and dual-strand assays for *CLIP4* (D), *KCNQ5* (E), and *C9orf50* (F). G, The sensitivity and specificity of the TriMeth test are shown for the combination of the 3 markers, based on either single- or dual-strand assays (samples were called positive or negative based on the 2-of-3 algorithm).

the cancer patients. Likewise, the *C9orf50* single-strand and *CLIP4* single- and dual-strand assays detected methylated DNA in the majority of CRC plasma samples (Fig. 4, A, B, and C). As expected, the *KCNQ5* and *CLIP4* dual-strand assays detected more methylated DNA copies in most of the CRC plasma samples compared with the single-strand assays (Fig. 4, A and B). The median signal fold-increase was 2.58 for the *KCNQ5* and 2.14 for *CLIP4*. In 1 patient with stage I CRC and 1 patients with stage II CRC, methylated DNA was detected by the *KCNQ5/C9orf50* single-strand assay but not by the corresponding dual-strand assay (Fig. 4, A and B; online Supplemental Table 4). However, only 1 methylated ctDNA molecule was detected in these samples. For samples with fewer than 6 methylated DNA molecules detected by the dual-strand *CLIP4* assay, it was not consistent whether the signal was higher for the dual-strand assay.

ROC curves illustrated how adding the antisense assay to the corresponding sense assay increased the area under the curve (AUC) of the *KCNQ5* marker (single-strand assay, AUC = 0.87; dual-strand assay, AUC = 0.95) and the *CLIP4* marker (single-strand assay, AUC = 0.87; dual-strand assay, AUC = 0.92) (Fig. 4, D and EF). The AUC of the *C9orf50* single-strand sense assay was 0.93 (Fig. 4, F). These results confirm the ability of the assays to discriminate plasma from CRC patients and healthy controls. After adding the 2 antisense assays, the new 3-marker test was called TriMeth-v2. Plasma samples were called positive or negative by applying a 2-of-3 algorithm as for the original test. With TriMeth-v2, the sensitivity increased from 74% to 86% and, importantly, the specificity remained 100% (Fig. 4, G).

The median ages of the cases and controls were 69 years (interquartile range, 66–79) and 70 years (interquartile range, 65–75), respectively. To further investigate the specificity of the TriMeth-v2 test, we also analyzed a cohort of 21 younger controls with a median age of 53 years (interquartile range, 50–55) (online Supplemental Table 1 and Supplemental Table 5). The analysis confirmed test specificity of 100% (online Supplemental Fig. 5).

Discussion

Blood-based detection of DNA methylation biomarkers holds great promise for early detection of cancer, but sensitivity is an issue. Highly sensitive assays are needed to detect the trace amounts of ctDNA present in early stage cancer patients. Many studies have used methylation-specific PCR-based detection strategies to detect ctDNA in plasma *(9, 19, 20)*. In this study, we developed methylation-specific ddPCR assays that simultaneously measured both the sense and the antisense DNA strands after bisulfite conversion to enhance the sensitivity for detection of DNA methylation markers in plasma from CRC patients. Assays targeting both DNA strands increased the number of methylated DNA copies detected when applied to methylated control DNA and CRC tissue and plasma samples from CRC patients. Furthermore, in comparison to measuring the sense strand only, dual-strand assays increased the sensitivity of the TriMeth test for detection of CRC-specific ctDNA in plasma from 74% to 86%; importantly, the dual-strand assay did not affect the test specificity, which remained 100% in the 42 examined healthy controls, spanning ages from 49 to 75 years. Consistently, the selected marker loci showed no correlation between methylation level and age (online Supplemental Fig. 1). The methylation level was also not affected by inflammation (online Supplemental Fig. 2).

The dual-strand assays quantified close to 100% of input methylated DNA, whereas the sense single-strand assays quantified approximately 50%. This finding supports the notion that DNA is single-stranded after cytosine conversion and that quantification can be improved by targeting both sense and antisense DNA strands. These results are consistent with the findings by Redshaw and colleagues *(21)* who showed amplification of 2 noncomplementary strands after bisulfite treatment and independent localization of these strands in separate partitions. In a screening setting, the test specificity is of utmost importance to avoid unnecessary and costly follow-up procedures. Therefore, we chose not to include the *C9orf50* antisense assay that showed some background signal. When the *CLIP4* and *KCNQ5* dual-strand assays were applied to plasma samples from CRC patients, they increased the signal by approximately 2-fold in all samples where >6 copies of methylated DNA were detected by the sense single-strand assay. For *CLIP4* and *KCNQ5*, measuring both DNA strands increased the AUC, probably owing to increased likelihood of detecting methylated ctDNA. For samples with <6 copies of methylated DNA detected with the sense single-strand assay, it was unclear whether the signal was increased by the dual-strand assays, probably because of subsampling challenges caused by very low levels of methylated DNA fragments in these samples. In early stage tumors, the number of ctDNA molecules in plasma may be very low, and it may become a question of chance whether the collected blood volume contains ctDNA. However, measuring both sense and antisense DNA strands will theoretically double the likelihood of detecting ctDNA in such a sample. The results from the present study demonstrate the advantage of measuring both sense and antisense DNA strands when using cytosine conversion-based approaches for detection of methylated DNA. The reported findings might be somewhat limited by the fact that CRC patients included in this study were diagnosed primarily based on symptoms. However, because we performed thorough testing of

our strategy in methylated control DNA and in CRC tissue and plasma from CRC patients, we assume that the results presented in this study would also apply to asymptomatic individuals.

In conclusion, this study showed that simultaneous measurement of both DNA strands after bisulfite conversion allows more sensitive detection of methylated DNA than measuring a single strand. Our work illustrates how the sensitivity of methylation-specific PCR-based methods can be improved. This approach may be particularly useful for liquid biopsy-based approaches for early blood-based detection of CRC.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations ctDNA, circulating tumor DNA CRC, colorectal cancer ddPCR, droplet digital PCR PBL, peripheral blood leukocyte AUC, area under the curvecirculating cell-free DNA.
Human Genes: *C9orf50*, chromosome 9 open reading frame 50; *KCNQ5*, potassium voltage-gated channel subfamily Q member 5; *CLIP4*, CAP-Gly domain containing linker protein family member 4.

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