Validation of a Circulating Tumor-Derived DNA Blood Test for Detection of Methylated BCAT1 and IKZF1 DNA

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Background: Colvera™ is a test that detects circulating tumor-derived DNA in patients with colorectal cancer by assaying for the presence of methylated BCAT1 and IKZF1 in blood. This study describes the analytical and clinical performance characteristics of the test.

Methods: Validation was performed in accordance with ISO15189 and National Pathology Accreditation Advisory Council requirements. Spiked samples including 264 plasma and 120 buffer samples were randomized, divided into 8 batches of 48 samples, and processed over 8 days using 2 equipment lines (each line consisting of a QIAsymphony SP/AS, QIAcube HT, and LC480); 2 reagent batches; and 2 operators to determine limit of detection, selectivity/specificity, precision, reproducibility, ruggedness, and susceptibility to commonly known interfering substances. Clinical performance was validated by assaying 222 archived plasma samples from subjects (n = 26 with cancer) enrolled in a previous prospective trial.

Results: The limit of detection for Colvera was 12.6 pg/mL (95% CI, 8.6–23.9 pg/mL), which equates to 2 diploid genome copies per milliliter plasma. No statistically significant difference was determined between testing days (n = 8), instrumentation, operators, or reagent batches in precision studies for the methylation-specific assays. The assay performance was unaffected by 9 commonly known interference substances, variations in bisulfite conversion, or quantitative PCR settings (cycling temperatures, incubation times, and oligonucleotide concentrations). For this clinical cohort, sensitivity and specificity estimates for Colvera were 73.1% (19 of 26; 95% CI, 52.2–88.4) and 89.3% (175 of 196; 95% CI, 84.1–93.2), respectively.

Conclusion: Colvera is a robust test and suitable for detection of circulating tumor-derived DNA by measuring levels of methylated BCAT1 and IKZF1 in human blood plasma.

IMPACT STATEMENT

Current noninvasive modalities for detection of colorectal cancer (CRC) have well-described deficiencies. We have developed a test (Colvera) that detects circulated tumor DNA by assaying for methylated BCAT1 and IKZF1 DNA in blood, which may provide an alternative aid in the detection of CRC. This study describes the analytical validation of Colvera and presents evidence that Colvera is a robust and automated test suitable for clinical testing.

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Colorectal cancer (CRC) is the second leading cause of death from cancer in the developed world. Early detection as a result of screening, advances in surgical techniques, and chemotherapy and radiotherapy have improved cure rates (1), but approximately 30% to 40% of patients with CRC will suffer recurrence despite achieving remission with initial treatment (2). Consequently, patients are entered into a follow-up regimen to detect recurrent disease at a stage where further curative-intent therapy is possible.

Primary and metastatic tumors release DNA into blood [circulating tumor DNA (ctDNA)], and it is well-documented that the ctDNA decreases after resection of tumor and increases as metastases develop (3–8). Hence, detection of ctDNA in bodily fluids such as blood plasma may have clinical utility as an aid for treatment of patients with cancer and as an indication of primary disease (9, 10).

ctDNA is commonly detected by targeting tumor-specific genetic or epigenetic alterations (11), and the most advanced clinical applications are using tumor-specific mutations to detect ctDNA for monitoring response to therapy and detection of minimal residual disease (12–16). However, large-scale collaborative sequencing projects such as The Cancer Genome Atlas and the International Cancer Genome Consortium have revealed that few mutations are observed in >5% to 10% of tumors of a particular tissue type (17). Further, mutation patterns are highly variable in genes with “hot spots” because of tumor heterogeneity (18). CRC has been reported to have a mutation frequency range of 0.1–100/Mb (18) with known hot-spot genes such as KRAS, BRAF, APC, and TP53 found to be mutated in only 15% to 40% of patients with CRC (17). The wide variability among mutation patterns has been addressed by development of individualized assays using a panel of genes (9, 18). Although these studies provide encouraging evidence of the ability to detect ctDNA using mutation-based panels, such individualized test methods are not easily implemented in an expanded patient population.

For a range of tumors, there is growing evidence that aberrant methylation shows less heterogeneity than most mutations (19, 20). As the methylation events are not confounded by the need to cover multiple and often large regions as is the case for mutations, methylation-based detection of ctDNA may be more applicable for monitoring of ctDNA dynamics (8).

We have previously reported that the promoter regions of the 2 genes BCAT1 and IKZF1 are hypermethylated with high frequency in CRC tissues compared with healthy colon tissues (21). Both BCAT1 and IKZF1 appear to be involved in tumor growth and invasiveness (22–27), and dysfunctional regulation of BCAT1 may induce chemoresistance to cisplatin (28). Several methods have been developed to detect ctDNA, e.g., NextGen sequencing, droplet digital quantitative PCR (qPCR), and “BEAMing” (5, 11), but we have developed a simple real-time qPCR assay for detection of methylated BCAT1 and IKZF1 DNA in blood, which detects ctDNA in patients with primary or recurrent CRC with an overall sensitivity for disease of 62–68% at an 88–94% specificity (29–33).

The aim of this study was to analytically and clinically validate the ctDNA blood test Colvera, which has been further optimized and automated on a commercially available platform to increase ease of use in a routine pathology diagnostic setting.

**MATERIALS AND METHODS**

**Study overview**

This work supports the validation of the ctDNA blood test, Colvera, consisting of a sample process
method using commercially available reagents and instrumentation. The test method extracts and bisulfite converts circulating cell-free DNA for subsequent detection of ctDNA by measuring the presence of methylated BCAT1 and IKZF1 DNA using a real-time qPCR-based method that is both specific and reproducible. The PCR target regions are as previously described (29), with the exception that the IKZF1 assay was modified to enable detection of partial methylation in 3 interprimer CpG sites within the target region (32). The analytical validation was performed using spiked plasma and buffer samples in accordance with the quality requirements as defined by the International Organization for Standardization (ISO 15189:2012) and the Australian National Pathology Accreditation Advisory Council guidelines. The clinical validation was performed using 222 archived K3-EDTA plasma samples (n = 26 with cancer) from a previous prospective study (30). All samples described herein were deidentified, stored at −80 °C as 4.5-mL aliquots in 5-mL Nalgene cryovials (Thermo Fisher, Victoria, Australia), and assayed by trained and qualified staff blinded to the clinical status of the samples.

In vitro samples

Bulk plasma pooled from gender-matched healthy donors <30 years of age was sourced through Bioreclamation (NY) and used unspiked (P0) or spiked with enzymatically fully methylated human genomic DNA (methylated CpGenome DNA; Merck-Millipore) at 500, 250, 100, 50, 25, 12.5, 6.3, 3.1, and 1.6 pg/mL (referred to as P500 to P1.6).

For interference testing, bulk plasma containing 500 pg/mL methylated CpGenome DNA was supplemented with the following substances (sourced through Sigma-Aldrich unless otherwise specified) to at least the upper limit of their corresponding reference ranges (34): 40 g/L albumin, 0.2 g/L bilirubin, 5 g/L cholesterol, 10 g/L D-(-)-glucose, 1 g/L hemoglobin, 20 g/L K3-EDTA, 0.4% (v/v) red blood cells, 12 g/L triglycerides, 0.235 g/L uric acid, or 100 ng/mL unmethylated human genomic DNA (Merck-Millipore).

Quality control materials and qPCR standards

PBS, pH 7.4 (Lonza), was supplemented with 5% (w/v) bovine serum albumin (Bovogen Biologicals) and 4 ng/mL unmethylated human genomic DNA to produce negative process control samples (NEGCONT). Positive process control samples (POSCONT) were further supplemented with 500 pg/mL methylated CpGenome DNA.

A 7-point, 2.5-fold standard dilution of bisulfite-converted methylated CpGenome DNA was included on each PCR plate (range: 2000 to 8.2 pg/well, triplicate input, 30 plates, n = 90 data points per concentration). Data graphs are available in Fig. 1 in the Data Supplement that accompanies the online version of this article at http://www.jalm.org/content/vol2/issue2 (R² values: ACTB, 0.9658; BCAT1, 0.9500; IKZF1, 0.9600), with good precision measured between 2000 and 128 pg/(<3% CV for each assay, see Table 1 in the online Data Supplement). The mass of methylated BCAT1 or IKZF1 DNA was expressed as the average mass (pg) of methylated BCAT1 or IKZF1 DNA per triplicate PCR assay.

Batch-specific acceptance criteria were pre-defined before the study for process controls and standards using a 99.7% CI from data generated before the study described herein.

Testing method

The QIAsymphony SP instrument (QIAGEN) was loaded with 4.5 mL of plasma in 5-mL Nalgene tubes, and cell-free DNA was extracted from 4-mL samples using the QIAsymphony Circulating Nucleic Acid kit (QIAGEN) according to manufacturer’s instructions [details available in Symonds et al. (32)]. The extracted DNA (approximately 85 μL) was bisulfite-converted using a thermal cycler (Axygen Maxygene) and repurified on a QIAcube
HT liquid handler (QIAGEN) using the Epitect Fast 96 Bisulfite Conversion kit per the manufacturer’s recommendation (QIAGEN) with the following adjustments/modifications: Buffer volumes were proportionately scaled to the 85-μL starting volume; a QIAamp filter plate was used instead of the Epitect filter plate; and the final plate drying and elution with 70 μL of nuclease-free water were performed by centrifugation rather than vacuum. The resulting bisulfite-converted DNA (approximately 42 μL) was analyzed as 3 replicates of 12 μL in a total PCR volume of 30 μL including 1× QuantiTect Multiplex PCR NoROX mastermix (QIAGEN) and oligonucleotides. The triplex real-time qPCR assay (ACTB quality control, methylated BCAT1 and IKZF1) was performed on an LC480 II (Roche Diagnostics). Cycle threshold (Ct) values were calculated using the absolute quantification second derivative algorithm provided with the LC480 software. The Colvera assay was qualitatively called “positive” if any methylated BCAT1 or IKZF1 was detected in any assay replicate within 50 PCR amplification cycles. No single-nucleotide polymorphisms that would affect primer or probe binding occur for any of the Colvera target genes (at >1% frequency; db single-nucleotide polymorphisms build 147 accessed via the UCSC Genome Browser at genome.ucsc.edu); thus, single-nucleotide polymorphisms pose no known false-negative issue for the Colvera assay. Oligonucleotide sequences and PCR cycling conditions are available in the article by Symonds et al. (32).

Analytical performance

For the analytical validation, the in vitro samples were divided into 8 batches of 48 and processed over 8 days by 2 different operators, using 2 different batches of reagents. All 8 batches contained equivalent sample types, but the order within each batch was randomized. Each batch contained 2 positive process control samples and 13 NEGCONT samples, 11 interference samples, and 22 spiked plasma samples to determine the analytical performance characteristics. The ruggedness of the bisulfite conversion step was determined using simulated DNA samples (2 ng of methylated CpGenome DNA in 32 ng of unmethylated human genomic DNA, 8 replicates/test parameter) to assess the effect of fluctuations in thermal cycling parameters (±1 °C and ±10% cycling time). The ruggedness of the qPCR cycling parameters and oligonucleotide concentration (±20%) was assessed as above using the qPCR standards.

Clinical performance

The clinical validation of Colvera was assessed using 4 mL of archived plasma specimens collected prospectively from patients scheduled for colonoscopy and enrolled under a previous prospective
study undertaken from September 2012 to May 2014 (30) and approved by the Southern Adelaide Clinical Human Research Ethics Committee. The trial is registered at the Australian and New Zealand Clinical Trial Register (ANZTR12611000318987). Representative plasma specimens of 2 phenotypes (primary CRC and nonneoplastic controls) were randomly selected with consideration of plasma availability and distribution of age and gender.

**Statistical analysis**

All analyses were conducted by use of Stata Version 13.1. The analytical precision of test results was analyzed using a 3-way ANOVA (no interactions included) with the following covariates: operators, reagents, and days. Matrix substance impact was acceptable if the 90% CI of two 1-sided tests (35) were within the equivalence margin, $\delta$ determined as $\delta = 1.5 \times \sigma$, and where $\sigma$ was the SD calculated from test results obtained from plasma spiked with 500 pg/mL methylated CpGenome DNA only (36). One-way ANOVA was used for assay ruggedness analysis. The relationship between concentration and test positivity was assessed by Probit regression modeling, and the limit of detection (LOD) was estimated as the concentration that resulted in 95% probability of determining a positive result. McNemar test (2-sided, significance level, 0.05) was used for paired positivity proportions and concordance analyses. Binomial distribution was assumed for calculations of exact 95% CI. $P$ values <0.05 were considered statistically significant.

**RESULTS**

The Colvera test is summarized in Fig. 1. In brief, samples were analyzed in batches of 48 including 44 samples and 2 positive process control samples and 2 NEGCONT samples, with test results reported within 14 h. Thirty PCR plates were run to process 606 in vitro and clinical samples (for all raw data, see Table 2 in the online Data Supplement).

![Fig. 2. Determination of the LOD.](image)

Process controls confirmed no confounding variation in batch processing, and the ACTB qPCR assay confirmed successful recovery of bisulfite-converted DNA from all processed samples (data not shown).

**Analytical sensitivity, linearity, and accuracy**

The LOD was determined using bulk plasma spiked with 7 concentrations of methylated CpGenome DNA (sample replicates per spike: 0 pg/mL, $n = 32$ and 1.6, 3.1, 6.3, 12.5, 25, and 50 pg/mL, $n = 8$). Based on a Probit regression model using all samples, the LOD$_{95}$ was estimated to be 12.6 pg/mL (95% CI, 8.6–23.9 pg/mL), which is the equivalent of 2 diploid genomes/mL of plasma (Fig. 2). Data graphs and accuracy tables for the
IKZF1 and BCAT1 qPCR methylation assays are available in Fig. 2 and Table 3 in the online Data Supplement.

**Analytical selectivity and specificity**

All sequenced PCR products showed a 100% match to the expected target sequences (3 PCR products for each target gene; data not shown).

**Reproducibility and repeatability**

Reproducibility and repeatability were determined by assaying plasma samples spiked with 0, 100, 250, and 500 pg/mL methylated DNA (n = 128, with 32 replicates per concentration). ACTB had a statistically significant difference for operator in the unspiked bulk plasma (P0), F(1,24) = 5.79, P = 0.0242 (Table 1) with a detectable difference of 0.85 Ct. There were no other significant differences between testing days (n = 8), operators, or different reagent batches (Table 1).

For the 4 replicates of each spike within a batch, the %CV was calculated from the average and SD of the Ct values for all positive replicates. The average %CV for the ACTB, BCAT1, and IKZF1 assay components was 1.7 (1.5–2.0), 1.9 (1.5–2.2), and 2.0 (1.6–2.5), respectively.

**Interference testing**

The impact of interfering substances on test performance was assessed by testing bulk plasma samples (containing 500 pg/mL methylated CpGenome DNA) with or without a potential interference substance (8 replicate samples per substance; Fig. 3). The test performance was not affected by any of the substances tested. The equivalence margin for ACTB was, as expected, exceeded by addition of 100 ng/mL unmethylated human genomic DNA and to a lesser extent by 0.4% (v/v) red blood cells, which were prepared from whole blood and therefore likely to contain a proportion of nucleated cells.

**Carryover**

The risk of process contamination was determined by assessing the methylation signal in 104 NEGCONT samples scrambled across the 8 validation batches (randomized sample positions, 13 sample replicates per batch). As expected, all NEGCONTs were positive for ACTB [mean Ct ± SD,}

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**Table 1. Reproducibility ANOVA results.**

<table>
<thead>
<tr>
<th>Assay</th>
<th>MetDNA a</th>
<th>Operator</th>
<th>Reagent</th>
<th>Day</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/mL</td>
<td>F (df1, df2)</td>
<td>P</td>
<td>F (df1, df2)</td>
<td>P</td>
</tr>
<tr>
<td>ACTB</td>
<td>0</td>
<td>5.79 (1, 24)</td>
<td>0.024</td>
<td>1.41 (1, 24)</td>
<td>0.247</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.89 (1, 24)</td>
<td>0.354</td>
<td>1.33 (1, 24)</td>
<td>0.260</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>1.84 (1, 24)</td>
<td>0.188</td>
<td>0.53 (1, 24)</td>
<td>0.472</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.00 (1, 24)</td>
<td>0.905</td>
<td>2.40 (1, 24)</td>
<td>0.134</td>
</tr>
<tr>
<td>BCAT1</td>
<td>100</td>
<td>0.14 (1, 24)</td>
<td>0.711</td>
<td>0.30 (1, 24)</td>
<td>0.590</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0.80 (1, 24)</td>
<td>0.381</td>
<td>0.94 (1, 24)</td>
<td>0.343</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.91 (1, 24)</td>
<td>0.349</td>
<td>0.17 (1, 24)</td>
<td>0.687</td>
</tr>
<tr>
<td>IKZF1</td>
<td>100</td>
<td>0.11 (1, 24)</td>
<td>0.738</td>
<td>1.11 (1, 24)</td>
<td>0.362</td>
</tr>
<tr>
<td></td>
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<td>0.496</td>
<td>0.00 (1, 24)</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>0.20 (1, 24)</td>
<td>0.656</td>
<td>0.22 (1, 24)</td>
<td>0.642</td>
</tr>
</tbody>
</table>

**a** MetDNA, concentration of spiked-in fully methylated DNA.

**b** Days 7 and 8 omitted because of collinearity.
34.2 (1.3); 95% CI, 34.1–34.3; 3.8% CV). In total, 310 of 312 PCR replicates (99.4%; 95% CI, 97.7–99.9) were negative for BCAT1 and IKZF1 methylation. The 2 positive replicates were from 2 different samples in 2 different batches wherein the remaining 12 NEGCONT samples were negative. This positivity may be because of spurious methylation in the spike used rather than contamination or carryover.

Assay ruggedness

Performance was not affected by temperature variations of ±1 °C or incubation times of ±10% in either the bisulfite conversion step (see Fig. 3 in the online Data Supplement) or real-time PCR step (see Fig. 4A in the online Data Supplement). Further ±20% variations in oligonucleotide concentrations did not impact assay performance (see Fig. 4B in the online Data Supplement).

Clinical performance

The clinical performance was assessed using archived plasma specimens previously tested and reported by Pedersen et al. (30). In brief, in that study, 129 patients with CRC and 1291 nonneoplastic patients, 33–85 years of age, were evaluated by colonoscopy. From those cases with sufficient plasma available, 222 specimens were randomly selected: 26 CRC cases and 196 nonneoplastic cases. The median age was 63 years (range, 45–85 years) with 62.2% female (see Table 4 in the online Data Supplement). Testing was completed as described for the analytical validation with technicians blinded to the clinical status.
All process controls and internal sample control \((\text{ACTB})\) were within predefined criteria ranges, hence deeming test results valid for analysis. Colvera was positive for \(\text{BCAT1}\) and/or \(\text{IKZF1}\) in 19 of 26 cancer cases with 62\% (95\% CI, 41–80) methylation positive for \(\text{BCAT1}\) and 54\% (95\% CI, 33–73) for \(\text{IKZF1}\) (58\% concordance), resulting in a 73\% (95\% CI, 52–88) agreement for the assay with clinical status. Of the 196 nonneoplastic cases, 22 were \(\text{BCAT1}\) and/or \(\text{IKZF1}\) positive with 8\% (95\% CI, 4–12) and 5\% (95\% CI, 2–9) methylation positive for \(\text{BCAT1}\) and \(\text{IKZF1}\), respectively (9\% concordance). This is an 89\% (95\% CI, 84–93) agreement with clinical status. The test positivity for cancers was statistically significant \((z\text{ score } t\text{-test}, P < 0.001)\).

**DISCUSSION**

The Colvera blood test detects ctDNA by assaying for the presence of methylated \(\text{BCAT1}\) and \(\text{IKZF1}\) DNA. In this study, the test was automated on commonly used commercially available instrumentation to reduce human error and to improve sample throughput. The qPCR assay was further optimized to enable detection of partial methylation in the targeted \(\text{IKZF1}\) region. Using spiked plasma and buffer samples, as well as archived plasma samples collected from a previous prospective study, we validated the Colvera test for detection of methylated \(\text{BCAT1}\) and \(\text{IKZF1}\) DNA in terms of LOD, specificity, imprecision, accuracy, linearity, interferences, ruggedness, carryover, and overall test result agreement with clinical status. The Colvera test processes 48 samples within 14 h when run in batch mode.

The test had an LOD of 12.6 pg/mL (approximately equivalent to 2 diploid genomes/mL of plasma) with a linear response between 25 and 500 pg/mL, and insignificant run-to-run, instrument-to-instrument, lot-to-lot, operator-to-operator, or intraassay variability [overall CV, 1.9\% (1.7–2.1)]. The observed operator-dependent variation in total DNA yield \((\text{ACTB})\) was deemed inconsequential, as this difference is likely to be because of a necessity to compromise PCR amplification efficiency for this control assay to ensure maximum sensitivity for the methylation DNA markers. The source of the observed 0.64\% positivity in con-

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**Fig. 4. Clinical validation.**

The Colvera qPCR Ct results \((y\text{ axis}; \text{mean Ct of 3 replicates})\) on 222 archived plasma samples from colonoscopy-confirmed patients (black circles, 196 nonneoplastic study participants; open circles, 26 patients with CRC) for the 3 assay components. \(\text{ACTB}\) (A), \(\text{BCAT1}\) (B), and \(\text{IKZF1}\) (C) were compared with the mean qPCR Ct values reported by Pedersen et al. \((x\text{ axis})\) (30). An arbitrary value of 50 was used for negative samples for graphical purposes.
control samples expected to be negative cannot be distinguished between operator-based contamination during process handling, intra-/interbatch carryover, and/or low-level methylation in the control human genomic DNA used. The test exhibited considerable ruggedness, as the performance was not compromised by minor thermal cycler variations during bisulfite conversion or by fluctuations in PCR cycling conditions or oligonucleotide concentrations during target detection and amplification. None of the tested interference substances commonly occurring in blood samples had an impact on detection of methylated BCAT1 and IKZF1 DNA.

An initial assessment of clinical performance was undertaken on 222 archived plasma samples from a previous study (30), and samples were selected without knowledge of the previous results. The true positive and negative rates were 73% and 89% for cancer (n = 26) and nonneoplastic controls (n = 196; P value, <0.001), respectively, with a total percentage agreement with clinical status of 87% (194 of 222; 95% CI, 82–91). The independent test results described herein and by Pedersen et al. (30) demonstrated an overall concordance of 85% (81% for cancer and 86% for nonneoplastic controls), indicating good reproducibility.

There was no significant difference in the clinical performance obtained in this study compared with the data reported by Pedersen et al. (30) (McNemar τ-test; 26 CRC, P = 0.375; 196 nonneoplastic controls, P = 0.087). Good correlation was observed on the average qPCR Ct values measured by the 2 test methods (R²: ACTB, 0.7738; BCAT1, 0.9035; IKZF1, 0.9005) with a higher ACTB yield observed in the Colvera method detailed in this article. The higher ACTB yield is likely a result of the optimized sample process.

The true- and false-positive rates of Colvera have subsequently been determined in an untested and independent cohort of 1381 volunteers, with an estimated sensitivity for the detection of primary CRC tumors of 62% (41 of 66; 95% CI, 49–74) and specificity of 92% (1207 of 1315; 95% CI, 90%–93%) (32).

The only similar methylation-based ctDNA blood test is the Food and Drug Administration-approved screening test Epi ProColon, which detects methylated SEPT9 (37). Epi ProColon returned a cancer sensitivity of 48.2% with a 91.5% specificity for primary CRC in a screening population (10). The Colvera test has yet to be tested in a true screening population. Measuring the levels of methylated BCAT1/IKZF1 and SEPT9 have been compared with carcinoembryonic antigen (CEA) for detection of recurrent CRC following initial treatment, and whereas Colvera was reported to be significantly more sensitive than CEA in this context [68% vs 32% (CEA cutoff, 5 ng/mL), n = 28] (33), no significant difference was observed between methylated SEPT9 and CEA levels [56.7% vs 40.0% (CEA cutoff, 3.5 ng/mL), n = 30] (38).

In summary, Colvera is a robust test that has been formatted for automated liquid handling systems using a routine pathology platform and commercially available reagents. The data presented herein confirm that Colvera is suitable for the intended use of the test, i.e., detection of circulating colorectal tumor-derived DNA by measuring the levels of methylated BCAT1 and IKZF1 DNA in human blood plasma.
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