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

David H. Murray,[1]*[†] Rohan T. Baker,[1][†] Snigdha Gaur,[1] Graeme P. Young,[2] and Susanne K. Pedersen[1]

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*[3] 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.

[1]Clinical Genomics Pty Ltd, North Ryde, New South Wales, Australia; [2]Flinders Centre for Innovation in Cancer, Flinders University, Adelaide, South Australia, Australia.

[†]D.H. Murray and R.T. Baker contributed equally to this work.

*Address correspondence to this author at: Clinical Genomics Pty Ltd, 11 Julius Ave., North Ryde, NSW 2113, Australia. Fax +61-2-98875300; e-mail david@clinicalgenomics.com.

Colorectal cancer (CRC)[4] 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 hotspot 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

[3] **Human genes:** *BCAT1*, branched-chain amino acid transaminase 1 gene; *IKZF1*, Ikaros-family zinc finger 1 gene; *ACTB*, beta-actin gene.
[4] **Nonstandard abbreviations:** CRC, colorectal cancer; ctDNA, circulating tumor DNA; qPCR, quantitative PCR; NEGCONT, negative process control samples; LOD, limit of detection; Ct, cycle threshold; CEA, carcinoembryonic antigen.

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).

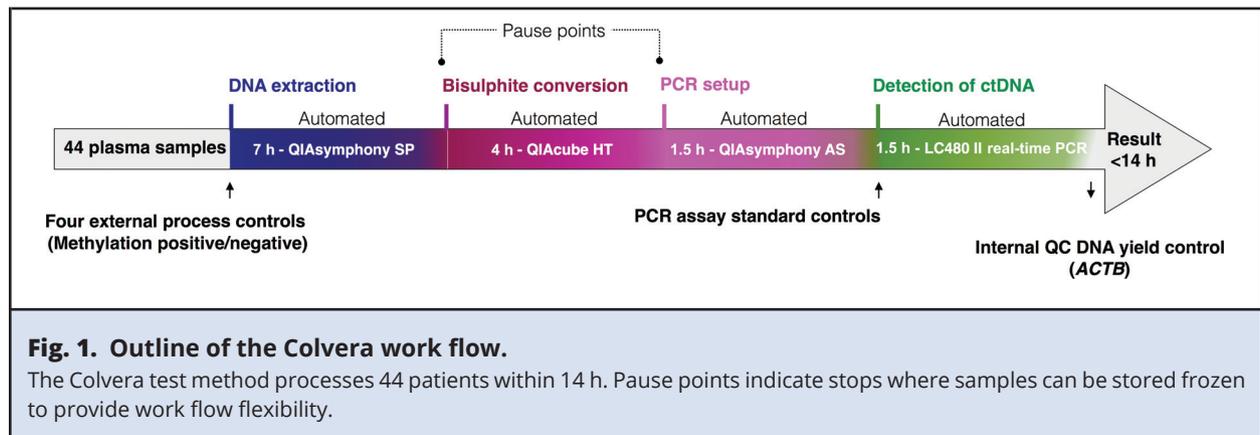### Quality control materials and qPCR standards

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 (R^2 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 predefined 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

Fig. 1. Outline of the Colvera work flow.
The Colvera test method processes 44 patients within 14 h. Pause points indicate stops where samples can be stored frozen to provide work flow flexibility.

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 condi-

tions 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, δ determined as $\delta = 1.5 \times \sigma$, and where σ 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).
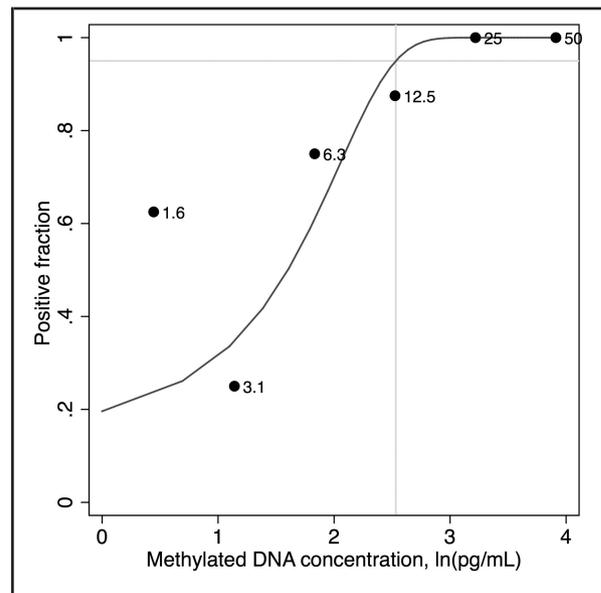

Fig. 2. Determination of the LOD.
The observed positive fractions of bulk plasma samples spiked with enzymatically methylated DNA (black circles, 8 sample replicates per concentration) were used to determine the LOD for Colvera. Gray line, estimated LOD (12.6 pg/mL; 95% CI, 8.6–23.9). The 0 pg/mL spike was included in the Probit regression model, but the data point is excluded in the figure, as the *x* axis is log-transformed.

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

Table 1. Reproducibility ANOVA results.

Assay	MetDNA[a] pg/mL	Operator F	(df₁, df₂)	P	Reagent F	(df₁, df₂)	P	Day F	(df₁, df₂)[b]	P	Model F	(df₁, df₂)	P
ACTB	0	5.79	(1, 24)	0.024	1.41	(1, 24)	0.247	2.52	(5, 24)	0.057	2.42	(7, 24)	0.050
	100	0.89	(1, 24)	0.354	1.33	(1, 24)	0.260	1.80	(5, 24)	0.152	1.89	(7, 24)	0.115
	250	1.84	(1, 24)	0.188	0.53	(1, 24)	0.472	0.41	(5, 24)	0.838	0.99	(7, 24)	0.461
	500	0.00	(1, 24)	0.905	2.40	(1, 24)	0.134	1.14	(5, 24)	0.367	1.12	(7, 24)	0.294
BCAT1	100	0.14	(1, 24)	0.711	0.30	(1, 24)	0.590	1.37	(5, 24)	0.271	1.38	(7, 24)	0.257
	250	0.80	(1, 24)	0.381	0.94	(1, 24)	0.343	0.32	(5, 24)	0.894	0.84	(7, 24)	0.562
	500	0.91	(1, 24)	0.349	0.17	(1, 24)	0.687	0.95	(5, 24)	0.469	0.82	(7, 24)	0.469
IKZF1	100	0.11	(1, 24)	0.738	1.11	(1, 24)	0.302	2.27	(5, 24)	0.079	2.21	(7, 24)	0.070
	250	0.48	(1, 24)	0.496	0.00	(1, 24)	0.988	0.59	(5, 24)	0.707	0.59	(7, 24)	0.707
	500	0.20	(1, 24)	0.656	0.22	(1, 24)	0.642	1.10	(5, 24)	0.387	0.82	(7, 24)	0.580

[a] MetDNA, concentration of spiked-in fully methylated DNA.
[b] Days 7 and 8 omitted because of collinearity.

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 resulting 80 comparisons was 1.9 (95% CI, 1.7–2.1), indicating that the within-batch variance was low at all concentration values. The
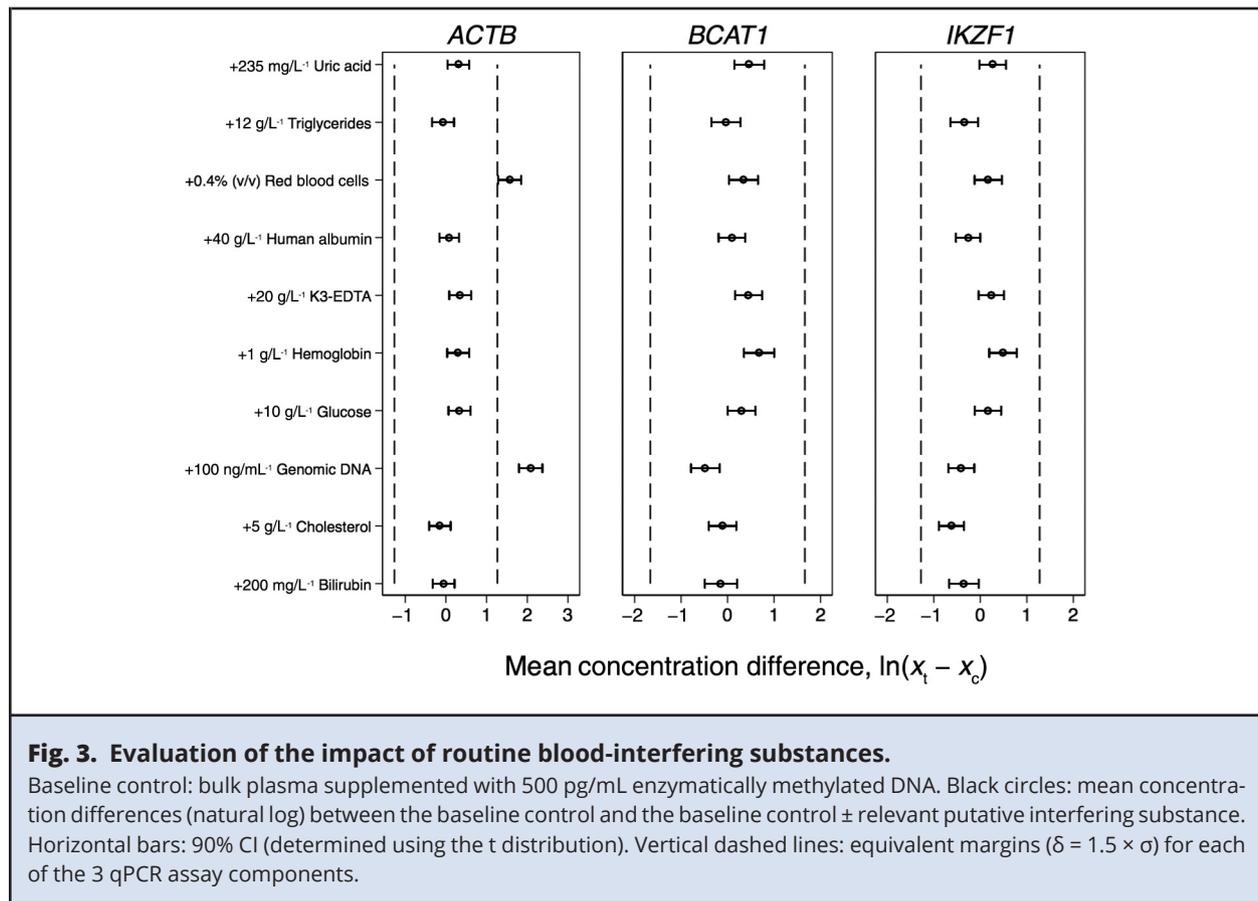
average %CV for the *ACTB*, *BCAT1*, and *IKZF1* assay components was 1.7 (1.5–2.0), 1.9 (1.5–2.2), and 2.0 (1.6–2.5), respectively.

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,

Fig. 3. Evaluation of the impact of routine blood-interfering substances.
Baseline control: bulk plasma supplemented with 500 pg/mL enzymatically methylated DNA. Black circles: mean concentration differences (natural log) between the baseline control and the baseline control ± relevant putative interfering substance. Horizontal bars: 90% CI (determined using the t distribution). Vertical dashed lines: equivalent margins (δ = 1.5 × σ) for each of the 3 qPCR assay components.

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 concentra-

tions 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.
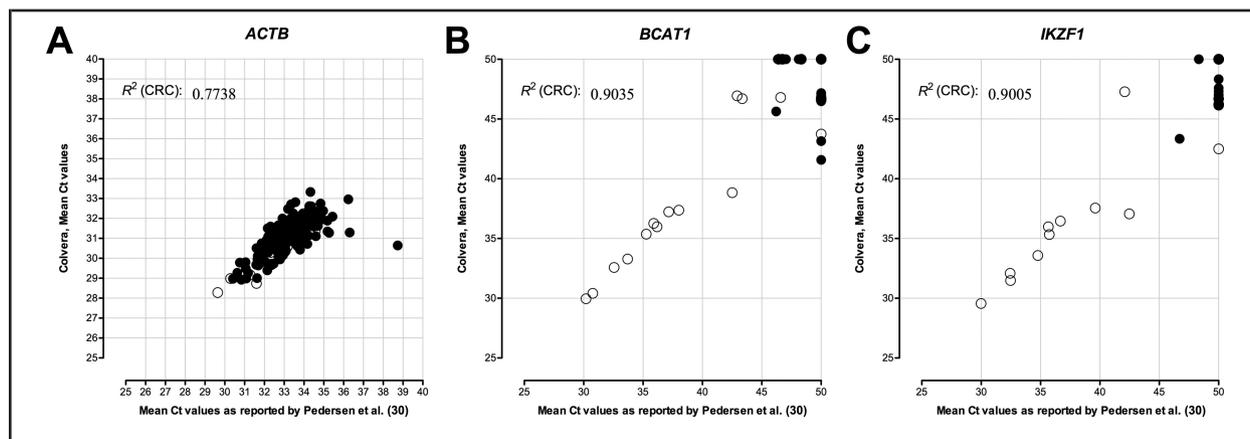
Fig. 4. Clinical validation.
The Colvera qPCR Ct results (y axis; mean Ct of 3 replicates) on 222 archived plasma samples from colonoscopy-confirmed patients (black circles, 196 nonneoplastic study participants; open circles, 26 patients with CRC) for the 3 assay components. *ACTB* (A), *BCAT1* (B), and *IKZF1* (C) were compared with the mean qPCR Ct values reported by Pedersen et al. (x axis) (*30*). An arbitrary value of 50 was used for negative samples for graphical purposes.

All process controls and internal sample control (*ACTB*) were within predefined criteria ranges, hence deeming test results valid for analysis. Colvera was positive for *BCAT1* and/or *IKZF1* in 19 of 26 cancer cases with 62% (95% CI, 41–80) methylation positive for *BCAT1* and 54% (95% CI, 33–73) for *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 *BCAT1* and/or *IKZF1* positive with 8% (95% CI, 4–12) and 5% (95% CI, 2–9) methylation positive for *BCAT1* and *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 score t-test, $P < 0.001$).

DISCUSSION

The Colvera blood test detects ctDNA by assaying for the presence of methylated *BCAT1* and *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 *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 *BCAT1* and *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 (*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-

trol 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*) (Fig. 4; McNemar *t*-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 (R^2: *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.

REFERENCES

1. Welch HG, Robertson DJ. Colorectal cancer on the decline—why screening can't explain it all. N Engl J Med 2016;374:1605–7.

2. Sargent D, Sobrero A, Grothey A, O'Connell MJ, Buyse M, Andre T, et al. Evidence for cure by adjuvant therapy in colon cancer: observations based on individual patient data from 20898 patients on 18 randomized trials. J Clin Oncol 2009;27:872–7.

3. Schwarzenbach H, Stoehlmacher J, Pantel K, Goekkurt E. Detection and monitoring of cell-free DNA in blood of patients with colorectal cancer. Ann N Y Acad Sci 2008; 1137:190–6.

4. Czeiger D, Shaked G, Eini H, Vered I, Belochitski O, Avriel A, et al. Measurement of circulating cell-free DNA levels by a new simple fluorescent test in patients with primary colorectal cancer. Am J Clin Pathol 2011;135:264–70.

5. Diehl F, Schmidt K, Choti MA, Romans K, Goodman S, Li M, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med 2008;14:985–90.

6. Newman AM, Bratman SV, To J, Wynne JF, Eclov NCW, Modlin LA, et al. An ultrasensitive method for quantitating circulating tumor DNA with broad patient coverage. Nat Med 2014;20:548–54.

7. Li M, Chen W-D, Papadopoulos N, Goodman SN, Bjerregaard NC, Laurberg S, et al. Sensitive digital quantification of DNA methylation in clinical samples. Nat Biotechnol 2009;27:858–63.

8. Garrigou S, Perkins G, Garlan F, Normand C, Didelot A, Le Corre D, et al. A study of hypermethylated circulating tumor DNA as a universal colorectal cancer biomarker. Clin Chem 2016;62:1129–39.

9. Tie J, Wang Y, Tomasetti C, Li L, Springer S, Kinde I, et al. Circulating tumor DNA analysis detects minimal residual disease and predicts recurrence in patients with stage II colon cancer. Sci Transl Med 2016;8:346ra92.

10. Church TR, Wandell M, Lofton-Day C, Mongin SJ, Burger M, Payne SR, et al. Prospective evaluation of methylated SEPT9 in plasma for detection of asymptomatic colorectal cancer. Gut 2014;63:317–25.

11. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer 2011;11:426–37.

12. Bettegowda C, Sausen M, Leary RJ, Kinde I, Wang Y, Agrawal N, et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. Sci Transl Med 2014;6:224ra24.

13. Bordi P, Del Re M, Danesi R, Tiseo M. Circulating DNA in diagnosis and monitoring EGFR gene mutations in advanced non-small cell lung cancer. Transl Lung Cancer Res 2015;4:584–97.

14. El Messaoudi S, Mouliere F, Du Manoir S, Bascoul-Mollevi C, Gillet B, Nouaille M, et al. Circulating DNA as a strong multimarker prognostic tool for metastatic colorectal cancer patient management care. Clin Cancer Res 2016; 22:3067–77.

15. Li Y, Fu X-H, Yuan J-Q, Yang Z-Y, Mao C, Dong X-M, et al. Colorectal cancer: using blood samples and tumor tissue to detect K-ras mutations. Expert Rev Anticancer Ther 2015;15:715–25.

16. Thierry AR, Mouliere F, El Messaoudi S, Mollevi C, Lopez-Crapez E, Rolet F, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med 2014;20:430–5.

17. Vogelstein B, Papadopoulos N, Velculescu VE, Zhou S, Diaz LA, Kinzler KW. Cancer genome landscapes. Science 2013;339:1546–58.

18. Lawrence MS, Stojanov P, Polak P, Kryukov GV, Cibulskis K, Sivachenko A, et al. Mutational heterogeneity in cancer and the search for new cancer-associated genes. Nature 2013;499:214–8.

19. Burrell RA, McGranahan N, Bartek J, Swanton C. The causes and consequences of genetic heterogeneity in cancer evolution. Nature 2013;501:338–45.

20. Weisenberger DJ. Characterizing DNA methylation alterations from The Cancer Genome Atlas. J Clin Invest 2014;124:17–23.

21. Mitchell SM, Ross JP, Drew HR, Ho T, Brown GS, Saunders NFW, et al. A panel of genes methylated with high frequency in colorectal cancer. BMC Cancer 2014;14:54.

22. Tonjes M, Barbus S, Park YJ, Wang W, Schlotter M,

Lindroth AM, et al. BCAT1 promotes cell proliferation through amino acid catabolism in gliomas carrying wild-type IDH1. Nat Med 2013;19:1–11.

23. Javierre BM, Rodriguez-Ubreva J, Al-Shahrour F, Corominas M, Grana O, Ciudad L, et al. Long-range epigenetic silencing associates with deregulation of Ikaros targets in colorectal cancer cells. Mol Cancer Res 2011;9:1139–51.

24. Yoshikawa R, Yanagi H, Shen C-S, Fujiwara Y, Noda M, Yagyu T, et al. ECA39 is a novel distant metastasis-related biomarker in colorectal cancer. World J Gastroenterol 2006;12:5884–9.

25. Iacobucci I, Storlazzi CT, Cilloni D, Lonetti A, Ottaviani E, Soverini S, et al. Identification and molecular characterization of recurrent genomic deletions on 7p12 in the IKZF1 gene in a large cohort of BCR-ABL1-positive acute lymphoblastic leukemia patients: on behalf of Gruppo Italiano Malattie Ematologiche dell'Adulto Acute Leukemia Working Party (GIMEMA AL WP). Blood 2009; 114:2159–67.

26. Malinge S, Thiollier C, Chlon TM, Dore LC, Diebold L, Bluteau O, et al. Ikaros inhibits megakaryopoiesis through functional interaction with GATA-1 and NOTCH signaling. Blood 2013;121:2440–51.

27. Bible E. Neuro-oncology: BCAT1 promotes cell proliferation in aggressive gliomas. Nat Rev Neurol 2013; 9:420.

28. Zheng Y-H, Hu W-J, Chen B-C, Grahn T-H-M, Zhao Y-R, Bao H-L, et al. BCAT1, a key prognostic predictor of hepatocellular carcinoma, promotes cell proliferation and induces chemoresistance to cisplatin. Liver Int 2016; 36:1836–47.

29. Pedersen SK, Baker RT, McEvoy A, Murray DH, Thomas M, Molloy PL, et al. A two-gene blood test for methylated DNA sensitive for colorectal cancer. PLoS One 2015;10: e0125041.

30. Pedersen SK, Symonds EL, Baker RT, Murray DH, McEvoy A, van Doorn SC, et al. Evaluation of an assay for methylated BCAT1 and IKZF1 in plasma for detection of colorectal neoplasia. BMC Cancer 2015;15:654.

31. Symonds EL, Pedersen S, Cole SR, Massolino J, Byrne D, Guy J, et al. Improving participation in colorectal cancer screening: a randomised controlled trial of sequential offers of faecal then blood based non-invasive tests. Asian Pac J Cancer Prev 2015;16:8455–60.

32. Symonds EL, Pedersen SK, Baker RT, Murray DH, Gaur S, Cole SR, et al. A blood test for methylated BCAT1 and IKZF1 vs. a fecal immunochemical test for detection of colorectal neoplasia. Clin Transl Gastroenterol 2016;7: e137.

33. Young GP, Pedersen SK, Mansfield S, Murray DH, Baker RT, Rabbitt P, et al. A cross-sectional study comparing a blood test for methylated BCAT1 and IKZF1 tumor-derived DNA with CEA for detection of recurrent colorectal cancer. Cancer Med 2016;5:2763–72.

34. Delahunt B, Baird R, editors. RCPA manual: manual of use and interpretation of pathology tests. 7th Ed. Surry Hills (NSW, Australia): Royal College of Pathologists of Australasia; 2015. www.rcpamanual.edu.au.

35. Tsong Y, Dong X, Shen M. Development of statistical methods for analytical similarity assessment. J Biopharm Stat 2017;27:197–205.

36. Limentani GB, Ringo MC, Ye F, Berquist ML, McSorley EO. Beyond the t-test: statistical equivalence testing. Anal Chem 2005;77:221–6A.

37. Potter NT, Hurban P, White MN, Whitlock KD, Lofton-Day CE, Tetzner R, et al. Validation of a real-time PCR-based qualitative assay for the detection of methylated SEPT9 DNA in human plasma. Clin Chem 2014;60:1183–91.

38. Tham C, Chew M, Soong R, Lim J, Ang M, Tang C, et al. Postoperative serum methylation levels of TAC1 and SEPT9 are independent predictors of recurrence and survival of patients with colorectal cancer. Cancer 2014; 120:3131–41.