

Epi proColon®

The Epi proColon test is a blood test for colorectal cancer screening.

epigenomics

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1—Executive Summary

Full implementation of colorectal cancer (CRC) screening could dramatically reduce the impact and costs of this disease. A broad base of evidence supports the benefits of CRC screening. Recommendations and guidelines published by organizations and specialty societies like the United States Preventive Services Task Force (USPSTF) and the American Cancer Society (ACS) strongly advocate for CRC screening. Additionally, national public awareness campaigns, state-sponsored programs and programmatic adherence activities by healthcare providers actively target and recruit screening-eligible women and men—despite these efforts, CRC screening rates remain sub-optimal with one in three screening-eligible Americans non-adherent to guidelines.¹ A new blood-based CRC screening test, Epi proColon®, (Epigenomics AG, sponsor) would provide an additional screening choice for those who are not being screened, and has the potential to improve participation in CRC screening programs, and thereby identify cancers at a treatable stage, saving lives.

CRC is the third most diagnosed cancer and cause of mortality in both women and men, and is projected to account for 8% of all diagnosed US cancers in 2014.¹ When CRC is detected at an early localized stage, the 5-year survival rate is 90%; with progression to regional disease, five year survival remains high at 69%, but when diagnosed at late stage when cancer has spread to distant organs, 5 year survival drops to 12%.¹ Given the long CRC development cycle (>10 years), the lack of symptoms during development, the known increased risk with age, and the potential for cure at localized and regional stage, CRC is an obvious candidate for screening programs. Colonoscopy and fecal occult blood testing are the most commonly used of the recommended screening methods and an estimated 65.1% of eligible women and men in the US meet guideline recommendations for screening. However, provider, institutional, patient and method-specific barriers impede program participation, such that 34.9% of the US population ages 50-75 years remains unscreened in accordance with USPSTF recommendations.^{1,2,3} As a consequence, 61% of CRC cases are diagnosed when the cancer has spread regionally or to distant organs when treatment is less successful.¹ Improving screening access and participation could help in shifting detection to earlier stages, further improving patient outcomes.

The Epi proColon test is an *in vitro* diagnostic PCR method for the qualitative detection of methylated Septin9 DNA in EDTA plasma derived from patient whole blood specimens. This blood-based colorectal cancer screening test was designed to improve screening participation via the ease and convenience enabled by blood testing. The test detects methylated DNA that has been shed into the bloodstream from CRC tumors in the proximal and distal colon as well as the rectum. Genetic and epigenetic changes contribute to the development of CRC. Epigenetic effects include changes in DNA methylation that occur early in carcinogenesis. These detectable changes are valuable indicators and biomarkers for early cancer detection.⁴ In colorectal cancer tissues, but not normal colon tissue, cytosine residues in the v2 region of the *SEPT9* gene become methylated. Using the Epi proColon test, DNA is extracted from plasma, treated with bisulfite to convert cytosine bases that are not methylated to uracils, and analyzed by Real-Time PCR amplification. This

technology enables detection of single copies of methylated *SEPT9* tumor DNA in a background of non-tumor DNA in plasma. A positive test is indicative of CRC, which should be confirmed by colonoscopy.^{6,7}

The Epi proColon test has been evaluated in two large, multicenter clinical trials. The first prospective trial included 7,941 men and women of average risk for CRC, ages 50-85, who were enrolled at 32 US and German sites. Clinical performance was validated in 1,544 of the trial participants, using colonoscopy as the reference method. All patients with CRC or advanced adenomas as well as subsets of patients with small polyps and patients with no evidence of disease were included in the study. The Epi proColon test showed a positivity in CRC of 68% (30/44) and positivity in non-CRC of 21% (318/1500), respectively. The tests detected 134/621 advanced adenomas, 87/435 polyps, and was positive for 97/444 persons with no evidence of disease (NED). Adjustment to the study design resulted in a clinical sensitivity for CRC of 68% (53 – 80%) and an adjusted estimate of clinical specificity of 80% (78 – 82%). The Epi proColon detected CRC at all stages and in both the distal and proximal colon and rectum.

In the second prospective multi-center trial requested by the FDA, the Epi proColon test was compared to the most widely-used US commercial FIT test. Results from both tests were compared to results obtained from colonoscopy. Three-hundred and one women and men, ages 50-85, who were of average-risk with no family or personal history of CRC were enrolled at 61 US clinical sites. One hundred and ninety-nine enrollees had no current cancer diagnosis pre-colonoscopy. Blood and fecal specimens were collected prior to preparation for colonoscopy. To enrich the trial with CRC patients, blood and fecal specimens from 102 patients diagnosed with CRC or of high suspicion of CRC were collected at least 10 days after screening colonoscopy, but prior to any CRC treatment. Fecal specimens were not available for 11 subjects. The Epi proColon and FIT tests obtained sensitivities of 73% (74/101) and 68% (66/97) respectively, and specificities of 82% (163/200) and 97% (188/193), respectively. By comparison of these results, the Epi proColon test was found to be statistically non-inferior to the FIT test with respect to sensitivity, but not specificity.

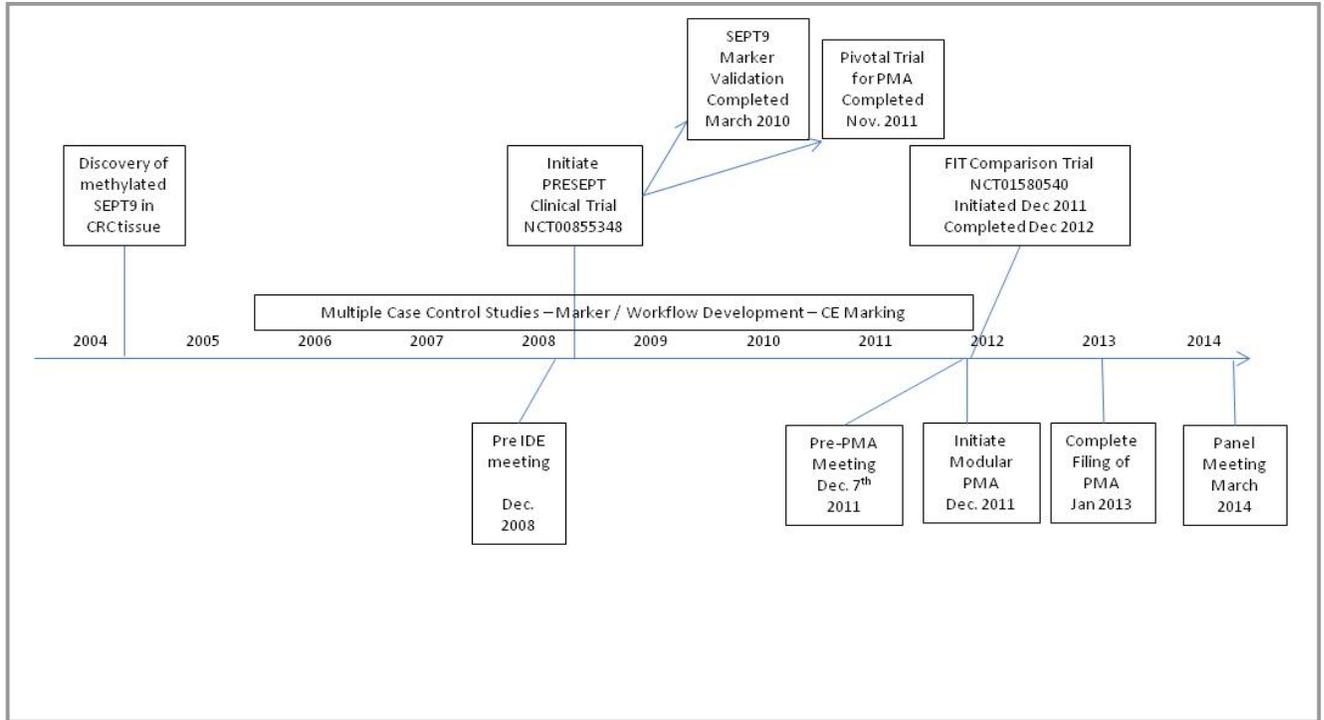


Figure 1-1: Key Milestones in the development of Epi proColon.

2—Background: Epidemiology and CRC Screening

Overview: There is a high potential for cure when CRC is diagnosed early. CRC screening may detect early stage cancers and adenomas while effective intervention and cure are still possible. The average-risk person may not experience symptoms or have indications of CRC; therefore, early detection is reliant on screening participation. Approximately 35% of persons who are defined as average-risk, age 50 and older do not participate in screening by recommended screening methods today. As a result, more than 60% of cases are diagnosed in later, symptomatic stages of disease resulting in poorer prognosis and outcome.

CRC Epidemiology

CRC is the third most common cancer, accounting for 8% of cancer-related deaths in the United States.¹ The cancer develops slowly, often over a period of 10-15 years, beginning with a non-cancerous tissue growth or polyp that develops in the lining of the colon or rectum.^{1,8} These adenomas occur in about 30 - 50% of adults age 50 years and older, but fewer than 10% of patients actually progress to cancer.^{1,5} CRC is associated with aging as 90% of cases are diagnosed in persons age 50 and older.^{1,5} The lifetime risk for being diagnosed with CRC is about 1 in 20 or 5% for both men and women in the US.

- Approximately 90% of diagnosed CRC cases and 94% of CRC-related deaths occur in men and women ages 50 years and older
- CRC incidence and mortality rates are highest in African American men and women

CRC Burden^{1,3}

ESTIMATED NEW COLORECTAL CANCER CASES AND CRC MORTALITY BY GENDER, 2014¹

	New Cases	Deaths
Men	71,830	26,270
Women	65,000	24,040

CRC Screening

Current options for screening include colonoscopy, flexible sigmoidoscopy, CT colonography, and gFOBT or iFOBT (FIT). For those that participate in screening, about 61.7% are screened by colonoscopy and 10.4% by fecal tests, for a combined test coverage of 65.1% (there is overlap in the methods used).² This translates into 34.9% or 23 million screening-age-eligible persons who are not up to date for screening with any of the USPSTF recommended tests for colorectal cancer screening.^{1,2} For those persons age of 75 and older, per USPSTF recommendation, the decision to screen should be based on an individual approach considering the person's

personal health history and risk.^{3,9,10} A good prognosis is directly related to early detection and the timing of diagnosis. When CRC is detected at an early, localized stage of disease, the 5-year survival rate is as high as 90%; however, only 39% of CRC patients are diagnosed at an early, localized stage, making more than 60% of CRC cases detected when CRC has spread locally or to distant organs.¹ With progression to regional disease, five year survival remains high at 69%, but the 5 year survival rate decreases to 12% for patients diagnosed with Stage IV distant metastases.¹ These data further illustrate the importance of screening in detecting CRC at a treatable stage.

Screening Methods: Current Recommendations

SCREENING GUIDELINES FOR AVERAGE-RISK INDIVIDUALS / CURRENT TEST CHOICES

Testing Method	American Cancer Society Age 50 ¹	National Comprehensive Cancer Network, Age 50 ¹¹	United States Preventive Services Task Force, Ages 50- 75 ³
FOBT	Yearly ^{*,**}	Yearly [†]	Yearly
FIT	Yearly ^{*,**}	Yearly [†]	Yearly
Stool DNA	Interval Uncertain	Not Recommended	Not Recommended
Sigmoidoscopy	Every 5 Years ^{**}	Every 5 years	Every 5 years [†]
Colonoscopy	Every 10 Years	Every 10 Years ^{†+}	Every 10 Years
CT Colonography	Every 10 Years ^{**}	Every 10 Years ⁺⁺⁺	Not Recommended

* FOBT/FIT when used as screening, multiple sample method must be used

** Colonoscopy should be done if these tests are positive

† A combination of sigmoidoscopy and annual fecal test every 5 years

† When performed with FOBT, FOBT repeated every 3 years

++ Preferred screening if available

+++ Patients with polyps >5mm should be referred for colonoscopy

2015 US Progress Towards Nationwide Goals to Reduce CRC

As reported by the American Cancer Society 2012 Strategic Plan Progress Report:¹³

- By 2013, reduce CRC incidence by 40% (From 1992-2007 there was a 21.9% reduction)
- By 2015, reduce mortality by 50% (From 1992-2007 there was a 30.4% reduction)
- Increase screening to 80% for those eligible (2008: NHIS, 53.2% for combined FOBT or endoscopy; 2008: BRFSS, 63.1%; 2010 BRFSS 65.4%, 2012 BRFSS 65.1%)
- Improve healthcare equity for the medical underserved by overcoming barriers that prevent screening

Barriers to Screening and the Medically Underserved

The reasons given for not participating in screening programs are well documented and include a lack of recommendation by healthcare providers, inconveniences, pain, fear and risks associated with endoscopy and anesthesia, discomfort with fecal collection and handling, financial and insurance coverage concerns, cultural beliefs, and lack or access to healthcare and preventive screening, among others.^{1,14}

Disparities in screening uptake by the medically underserved and in racial and ethnic minorities have been widely reported in literature. These occur in populations having a lower socioeconomic status, lower education

level, poor health literacy, in certain racial and ethnic groups and in those without insurance or a regular healthcare provider. Racial and ethnic minorities and poor people face more barriers to receiving preventive care, even with physician recommendation for screening by current methods. The adherence rate in African Americans remains low, ranging from 10% to 50%.^{15,16,22,23} African Americans have a 20% higher incidence and 45% higher mortality from CRC than Caucasians.²²

3— Background: CRC Biology, DNA Methylation, Methylated Septin9 DNA Biomarker

Overview: DNA methylation is an important epigenetic event that occurs in human cancers and may provide diagnostic and prognostic information for clinical decision-making. Using genome-wide discovery methods, Epigenomics identified methylated Septin9 as a discriminating plasma biomarker for CRC and developed and validated the Epi proColon blood-based test to detect this marker.

Colorectal Cancer Biology

- Normal epithelial cells transform into cancer cells due to reprogramming of critical biological processes resulting from DNA mutations and/or epigenetic changes within the cells^{16,17}
- Cell growth becomes uncontrolled and limitless, proliferating and invading adjacent and distant tissues, resulting in metastasis¹⁷⁻¹⁹
- A small percentage of cases are attributed to an inherited genetic defect — examples include familial adenomatous polyposis (FAP), hereditary non-polyposis colorectal cancer (HNPCC) or Lynch syndrome
- The majority of CRC cases (~75%) are sporadic and result from an accumulation of molecular events that affect the regulation of a set of genes involved in maintaining cell cycle control
- CRC is a heterogeneous disease with 3 molecular classification types: Microsatellite instability (MSI), Chromosomal instability (CIN), and CPG island methylator phenotype (CIMP)
- Concurrent hypermethylation changes in many CpG islands are associated with CIMP and ~20% of CRCs¹⁹

Figure 3-1: Colorectal Cancer Progression Pathway

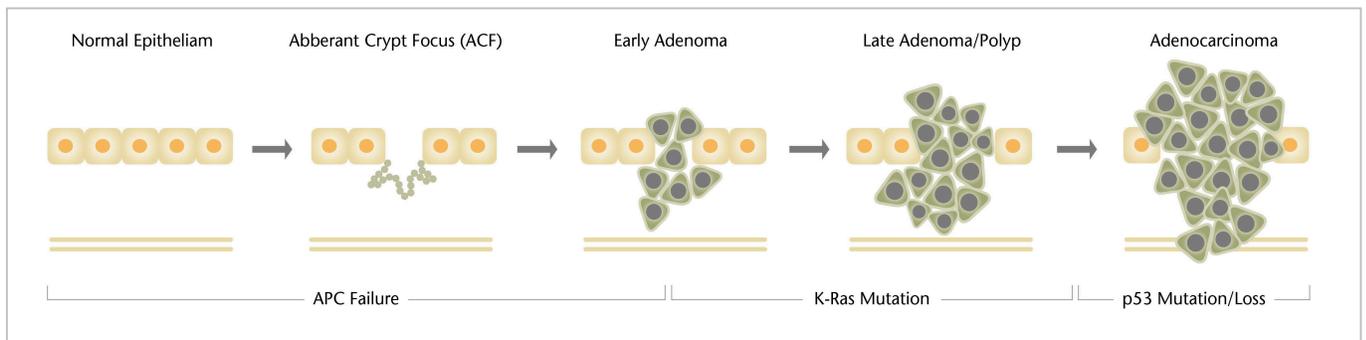


Figure 3-2: Commonly Methylated and Silence Genes in Colorectal Cancer

Commonly Methylated and Silenced Genes in Colorectal Cancer							
Specific genes and loci become aberrantly methylated at various steps of the ACF to adenocarcinoma sequence; these staged alterations contribute to both the initiation and progression of cancers.							
APC	CDKN2A/p16	DAPK	ID4	MINT1*	RASSF1A	SFRP1	TIMP3
CDH1	CRABP	ESR1	IRF8	MINT31*	RUNx3	SFRP2	THBS1/TSP1
CDH13	CXCL12	HLTF	MGMT	MLH1	SEPT9	SLC5A8	VIM

Lao, VV. Epigenetics and Colorectal Cancer. Nat Rev Gastroenterol Hepatol, 2011.
 *Loci found to be methylated in tumor; not genes

- Molecular events that disrupt gene regulation include DNA methylation
 Aberrant DNA methylation is the most extensively studied epigenetic mechanism in CRC

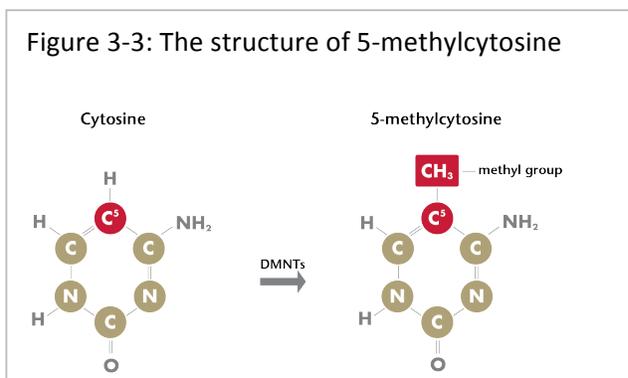
DNA Methylation and Cancer¹⁷⁻¹⁹

Cancer is both a genetic and epigenetic disease:

- Epigenetics refers to stable, heritable alterations in gene expression potential that occur during cell development and proliferation, that are not mediated by changes in the DNA sequence
- When individual genes or DNA become altered, a complex array of epigenetic regulatory mechanisms that control gene expression in both normal and cancerous cells are affected
- These detectable changes are valuable indicators and biomarkers for early cancer detection

DNA Methylation is a normal process that is essential for cell differentiation and embryonic development:

- DNA methylation in mammals is the modification of cytosine to 5-methyl cytosine by DNA methyl transferase (DNMT)
- Affects cytosines occurring as CpG dinucleotides
- Occurs in regions of high CG-base content, the CpG islands, which are often located in the 5' regulatory region of genes



Aberrant DNA methylation is an important epigenetic event:

- Occurs early in the development of human cancers
- Can provide critical diagnostic and prognostic information important for clinical decision making
- Associated with cancers in two forms, hypomethylation and hypermethylation:
 - Hypermethylation typically occurs at CpG islands in the promoter regions of genes, and is associated with gene silencing (inactivation)
 - Global hypomethylation is an early event in carcinogenesis that plays a role in both the development and progression of cancer (genomic instability)

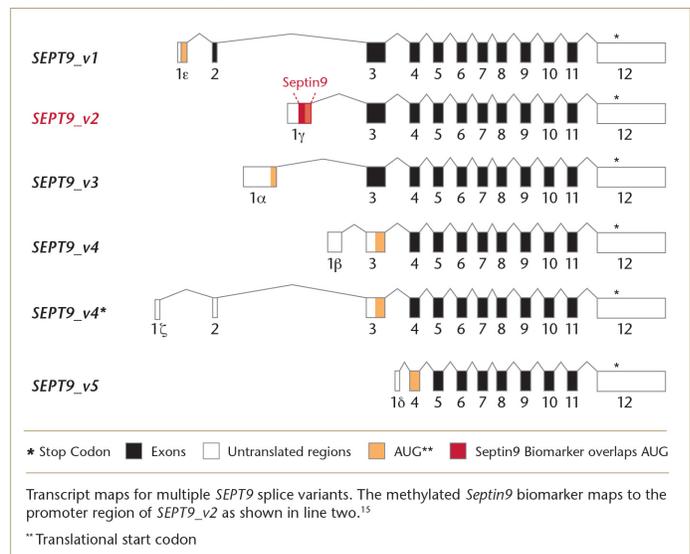
DNA Methylation Biomarkers

- Changes in DNA methylation are often markers for early events in carcinogenesis and precancerous lesions
- DNA methylation biomarkers have broad potential in cancer diagnostics and therapeutics⁴

Methylated Septin9 DNA Biomarker

- Genome-wide discovery methods were used to identify and characterize hundreds of DNA methylation-based biomarkers to discriminate CRC tumor DNA from DNA extracted from normal epithelia⁷
- The gamma promoter region of the v2 SEPT9 transcript was shown to be differentially methylated in CRC tissue⁷
- The SEPT9 sequence was methylated in >90% of CRC tissues⁷
- Real-Time PCR assays were developed using bisulfite treatment and the Company’s proprietary HeavyMethyl® PCR technology
- Through independent testing of multiple patient plasma sets, the v2 promoter methylation had the best discrimination for CRC detection (ROC AUC=0.92)⁷
- The specific role of SEPT9 in CRC is not known but hypermethylation of this sequence is associated with malignant transformation in CRC^{20,21}
- Not all CRC tumors shed methylated SEPT9 DNA into the bloodstream⁷

Figure 3-4: The Septin9 Gene Family



- Detection of methylated Septin9 is dependent on the amount of free circulating tumor DNA in the specimen and may be affected by sample collection methods, sample storage, patient factors and tumor stage⁶

The development of an assay for the measurement of methylated *SEPT9* in plasma required improved methods for extraction of cell free DNA, bisulfite conversion of the DNA and purification of the converted DNA (bisDNA), and a high sensitivity real time PCR assay for the detection of the target sequence. The development occurred through an iterative process in which incremental improvements were tested in independent case-control studies.²¹

Table 3-1: Case control studies for methylated Septin9²¹

Study	Samples	Sensitivity	Specificity	Workflow
1	312	52	95	Experimental Workflow
2	600	57	96	
3	725	48	93	
4	370	48	96	
5	550	72	90	
6	269	73	93	^m SEPT9 Detection Assay
7	245	69	89	

PRESEPT – Multicenter Prospective Clinical Trial in the Screening Population

Based on the performance of the methylated Septin9 biomarker in case-control studies cited above, Epigenomics sponsored the PRESEPT clinical trial to prospectively collect plasma from the screening-eligible population and determine the performance of the Septin9 biomarker in this setting (ClinicalTrials.gov, Trial Registration ID: NCT00855348). The study was designed to recruit subjects reporting for screening colonoscopy based on the US Multi-Society Task Force Guideline (USMSTF) screening criteria¹² (people of average-risk, age 50 and older). Prior to screening colonoscopy, each subject provided approximately 40 mLs of blood from which approximately 20 mLs of plasma were prepared and archived at -80°C to allow for multiple separate analyses of biomarkers and tests.

7,941 subjects were enrolled at 32 sites in the US and Germany from June 2008 through January 2010. Enrollment was closed in January 2010 when the goal of enrolling at least 50 CRC cases identified by screening colonoscopy and verification by clinical and histopathological examination was reached. Nearly 75% of the

patients were enrolled at one of 22 clinical sites in the US, and about 25% of the patients were enrolled at one of 10 clinical sites in Germany. Demographic data on subject age, gender, and ethnicity were recorded.

As part of the PRESEPT trial, a prospective evaluation study of the Septin9 biomarker using an early version of the Septin9 assay was conducted under the direction of the principal investigator (T. R. Church, University of Minnesota School of Public Health). This evaluation was based on a subset of PRESEPT plasma samples that included all cancer patients and a proportional random selection of samples from the other clinical sub-categories. As reported by Church et. al., the original assay had a crude cancer detection rate of 50.9% at 90.4% specificity.

The outcome of the PRESEPT study was the observation that methylated Septin9 was a viable biomarker for CRC screening.

4—The Epi proColon® Test: Test Summary

Overview:

The Epi proColon test is an *in vitro* polymerase chain reaction (PCR) assay for the qualitative detection of methylated Septin9 DNA isolated from 3.5 mL of patient plasma. The Epi proColon test comprises the Epi proColon Plasma Quick Kit, Epi proColon PCR Kit and Epi proColon Controls Kit. The test kits provide sufficient reagents to process 30 patient samples and 2 controls. The Epi proColon test detects methylated Septin9 DNA in EDTA plasma derived from patient blood specimens. The Epi proColon test reports to the physician qualitative Positive and Negative results only. The proposed interval of use for the test is annual testing. Detection of CRC DNA in plasma using the methylated Septin9 DNA biomarker has been demonstrated in multiple case-control studies of CRC patients and colonoscopy-verified negative controls. The Epi proColon test has been evaluated in two multicenter clinical studies. The blood-based Epi proColon test offers patients an alternative test option to participate in a CRC screening program, but is not intended to replace colonoscopy.

Proposed Intended Use

The Epi proColon test is a qualitative *in vitro* diagnostic test for the detection of methylated Septin9 DNA in EDTA plasma derived from patient whole blood specimens. Methylation of the target DNA sequence in the promoter region of the *SEPT9_v2* transcript has been associated with the occurrence of colorectal cancer (CRC)¹. The test uses a real-time polymerase chain reaction (PCR) with a fluorescent hydrolysis probe for the methylation specific detection of the Septin9 DNA target.

The test is indicated to screen patients for colorectal cancer who are defined as average risk for colorectal cancer (CRC) by current CRC screening guidelines. Patients with a positive Epi proColon test result should be referred for diagnostic colonoscopy. Men and women 50 to 85 years of age were included in the Epi proColon clinical trial. The Epi proColon test results, together with the physician's assessment of history, other risk factors, and professional guidelines, may be used to guide patient management.

The Epi proColon test is for use with the Applied Biosystems 7500 Fast Dx Real-Time PCR Instrument.

Proposed Warnings

- The Epi proColon test is not intended to replace colorectal screening by colonoscopy.
- The Epi proColon test is not intended to screen persons under the age 50 who are considered to be at average risk for colorectal cancer.
- Positive Epi proColon test results are not confirmatory evidence for the presence of colorectal cancer. Patients with a positive Epi proColon test result should be referred for diagnostic colonoscopy.

- A negative Epi proColon test result does not guarantee absence of cancer. Patients with a negative Epi proColon test result should be advised to continue participating in a colorectal cancer screening program that also includes colonoscopy, fecal tests and/or other recommended screening methods.
- Positive test results have been observed in clinically diagnosed patients with chronic gastritis, lung cancer and also in pregnant women

Utilization: Healthcare Provider—Patient Interface

Healthcare Provider-Patient Interface

- An individual approach that considers the patient’s personal health and family history, and preference is the focus of a discussion between the patient and healthcare provider. Physician recommendation and shared decision-making that involves the patient in their healthcare choices have been shown to be key factors contributing to greater screening participation.
- For average-risk patients age 50 and older, the Epi proColon test offers another test choice to the current menu of colorectal cancer screening tests that include fecal blood tests, colonoscopy, and other imaging tests.
- For patients who resist or refuse current recommended CRC screening methods including colonoscopy, and who otherwise may choose not to participate in screening programs, a blood test may encourage screening participation, and improve adherence and patient outcomes (as compared to no screening).

Getting Tested with the Epi proColon Test

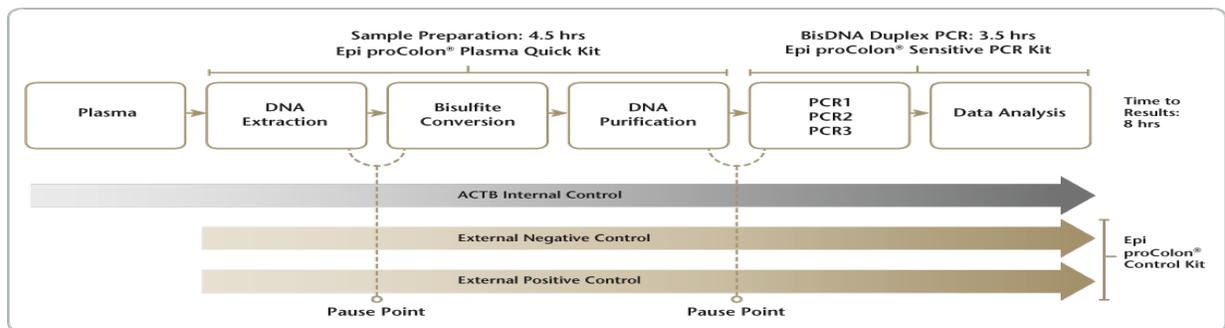
- A blood specimen may be drawn at a physician or healthcare provider office laboratory, reference laboratory draw station, or hospital laboratory (blood collection tube requirement: 10mL Vacutainer® K₂EDTA ONLY, by Becton Dickinson)
- Plasma preparation is performed at the drawing site within 4 hours of blood draw and the plasma is stored at 2° to 8°C for up to 72 hours or frozen at -15°C to -25°C for up to 14 days
- Epi proColon test results will be returned to the physician, when completed; results are reported as ‘Positive’ or ‘Negative’
- The physician (healthcare provider) will discuss test results with the patient and decide what the next appropriate steps are, based on the test results and the patient’s individual health history

Although longitudinal performance over time has not been established for the Epi proColon test, it is expected this would be similar to FIT and superior to stool guaiac test (gFOBT). Based on what is known about these comparators, annual testing is recommended.

Utilization: Laboratory Workflow

- The servicing laboratory for the physician or healthcare provider will provide test requirements and instructions to all draw stations that would collect blood from patients for this test
- Only 10mL BD Vacutainer K₂EDTA blood collection tubes are acceptable for blood collection (Becton Dickinson)
- Plasma preparation should be performed within 4 hours of blood draw
- Training materials for blood collection and processing have been developed for laboratories and draw stations

Figure 4-1 Overview of the Laboratory Test Workflow



The Epi proColon Test Real– Time PCR Overview: Two Procedural Phases

Phase 1

DNA is extracted from plasma, treated with bisulfite to obtain bisulfite-converted DNA (bis-DNA), then re-purified to obtain highly pure DNA.

Note: Bisulfite conversion is the choice method for analyzing DNA methylation.

Phase 2

Bis-DNA is assayed by duplexed Real-Time PCR with simultaneous detection of the target methylated Septin9 DNA and the internal control ACTB (β -Actin) DNA.

Notes:

- For this test, Real-Time PCR is performed in triplicate - for each sample, there are 3 PCR results analyzed.
- For every PCR well, the duplexed detection of bisulfite-converted ACTB (β -actin) DNA serves as an internal control. This co-amplified sequence provides a monitor for sample quality, sample preparation and adequate DNA concentration of the specimen.

Results Interpretation and Reporting

Assessment of the Validity of the Run by Epi proColon Controls

- Any run processed with the Epi proColon POSITIVE Control and Epi proColon NEGATIVE Control is considered VALID when adjacent criteria are met for **ALL THREE (3)** PCR replicates per control
- If either the Epi proColon POSITIVE Control or Epi proColon NEGATIVE Control, or both are INVALID, the data for patient specimens cannot be interpreted and testing must be repeated for all patient specimens included in this test run

Validity Limits of Epi proColon Control Kit

Result of Control	Determination	Septin9 Result	ACTB Result
Positive Control VALID	PCR1	Ct* ≤ 41.1	Ct* ≤ 29.8
	PCR2	Ct* ≤ 41.1	Ct* ≤ 29.8
	PCR3	Ct* ≤ 41.1	Ct* ≤ 29.8
Negative Control VALID	PCR1	No Ct* result ("Undetermined")	Ct* ≤ 37.2
	PCR2		Ct* ≤ 37.2
	PCR3		Ct* ≤ 37.2

* cycle threshold

Assessment of the Validity of a Single PCR of a Patient Specimen

- Provided that the result of the internal control assay ACTB indicates sufficient input of bis-DNA into the single PCR i.e., ACTB cycle threshold (Ct) at most 32.1, the result of the Septin9 PCR defines the result for this single PCR
- An ACTB cycle threshold above 32.1 turns the single PCR result to "INVALID PCR"

Interpretation of Results for Single PCR

Single PCR Result	Septin9 Result	ACTB Result
Septin9 Positive	Ct* < 45	Ct* ≤ 32.1
Septin9 Negative	No Ct* result ("Undetermined")	Ct* ≤ 32.1
INVALID	Any result	Ct* > 32.1 Or "Undetermined"

* cycle threshold

Interpretation of Results for a Patient Specimen

- The test result for a patient sample is "POSITIVE", if at least one PCR replicate is a Septin9 Positive PCR
- The test result for a patient specimen is "NEGATIVE", if all three PCR replicates are Septin9 Negative PCRs
- The test result is "INVALID" in all other cases

Interpretation of Epi proColon Test Results

Test Result	Positive & Negative Control	Single PCR Result
POSITIVE	VALID	At least one Septin9 Positive PCR [†]
NEGATIVE	VALID	PCR1: Septin9 Negative PCR PCR2: Septin9 Negative PCR PCR3: Septin9 Negative PCR
INVALID	VALID	All other cases [‡]
INVALID	INVALID	n/a

[†] One single PCR result is Septin9 Positive; the two remaining single PCR results may have any result (INVALID, Septin9 Negative, or Septin9 Positive)

[‡] No single PCR result is Septin9 Positive, at least one single PCR result is INVALID, the remaining single PCR results may be INVALID or Septin9 Negative.

5—Analytical Validation

Overview:

The analytical performance of the Epi proColon test was determined at four laboratories. The limit of detection (LoD) for methylated Septin9 was determined to be 4.7 pg/mL of plasma. The PCR cut off was established at 45 cycles. In repeatability and reproducibility studies, the coefficients of variation were estimated below 6% (on cycle threshold scale) for all 14 plasma pools tested. Tests showed no interference in test performance from substances commonly found in blood samples.

Analytical Sensitivity - Limit of Detection

The Limit of detection (LoD) of the Epi proColon test was determined at four laboratories according to CLSI EP12 and EP17 guidance documents. All sites utilized the test kit manufactured under final manufacturing conditions. In total, nine levels of technical samples were repeatedly tested with a range from 0 (blank) to 50 pg/mL of human DNA methylated at Septin9. The methylated DNA was spiked into human plasma and into an artificial matrix of Tris buffer plus BSA. The limit of detection of 4.7 pg/mL (95% CI, 2.5 - 9.0 pg/mL) for methylated Septin9 in plasma was estimated with logistic regression methodology.

Analytical Specificity / Cross-Reactivity

The specificity of the assay was determined in multiple ways. *In silico* analysis using blast and e-PCR revealed no cross reactivity of the Epi proColon oligonucleotides with the bisulfite converted human genome. In addition, repeated testing of negative controls or technical samples with genomic DNA not methylated at the Septin9 target locus yielded results that were consistently negative. An additional observation was that overloading samples with genomic DNA did not result in positive Septin9 signals. Finally, for a subset of non-CRC samples that tested positive with the Epi proColon test, sequencing confirmed methylation of the Septin9 PCR target area in all cases, demonstrating that the Epi proColon test detects true biological status rather than reporting cross-reactivity. In combination, these results indicate that the Epi proColon PCR assay is highly specific for the methylated Septin9 target.

Precision and Accuracy

Accuracy – Cut-off Establishment

The assay Ct thresholds for the Epi proColon test were established in a study performed using 156 clinical plasma samples (reference method: colonoscopy). The instrumentation was set to 50 cycles in order to establish the Septin9 and ACTB cycle threshold (Ct) limits on these clinical specimens. From the distributions of these data, it was evident that Ct values larger than 40 for Septin9 PCR are rare and no Ct-values are observed above a Ct of 45. The vast majority of ACTB measurements range between 26 to 30 Ct. There was a general observation in these clinical specimens of outliers having lower Ct-values. For very few specimens the ACTB

value came close to Ct of 31. From these data Septin9 and ACTB Ct limits of 45 and 32.1, respectively, were determined.

Accuracy – Cut-off Verification

A second study was performed using 346 clinical plasma samples. The instrumentation was set at 50 cycles for a subset of 197 plasma samples in order to verify the Septin9 and ACTB cycle threshold limits of 45 and 32.1, respectively, on these clinical specimens. The distributions of these data confirmed the results from the first study. Therefore, the cycle threshold limits were verified. For these 346 clinical samples, the overall agreement with clinical status was observed at 94.9% (95% CI 88.6 – 97.8%) for CRC cases, and 84.3% (95% CI 79.2 – 88.3%) for colonoscopy-verified controls.

Repeatability, Intermediate Precision and Reproducibility

To evaluate repeatability and intermediate precision of the assay, aliquots from 14 clinical sample pools were tested at three testing sites by six operators with three reagent lots using three PCR instruments. Each plasma pool was tested 12 times. Pools 1 – 6 generated from CRC plasma were positive in all 12 tests. For the three pools representing self-declared healthy blood donors, pool 7, pools 8 and pool 9 were each negative in 9 out of 12 tests. For the pools derived by diluting a single CRC plasma aliquot in human plasma, pools 10, 13, and 14 were positive in 11 out of 12 tests, while pools 11 and 12 were positive in all 12 replicates. In total, for 129 out of 132 samples where CRC plasma was tested (pools 1 – 6, pools 10 – 14), the test result was positive leading to 98% (95% CI 94 – 99%) positive percent agreement with clinical status. Aggregated over the three pools derived from healthy blood donors (pools 7 – 9), the test results for 27 out of 36 samples was negative leading to 75% (95% CI 59 – 86%) negative percent agreement with clinical status. The total percent agreement estimated from these data is 156/168, i.e. 93% (95% CI 88 – 96%). There were no differences in the positive and negative percent agreement attributable to sites, operators or kit lots.

In addition, precision and reproducibility analyses were conducted with Ct values generated on the set of 14 sample pools. For precision, the ranges of standard deviation and coefficient of variation for Septin9 were 0.4 – 2.1 Ct and 1.1 – 5.5%, respectively. The corresponding ranges for ACTB were 0.2 – 0.4 Ct and 0.8 – 1.7%.

For reproducibility, the ranges of standard deviation and coefficient of variation for Septin9 were on 0.4 – 2.3 Ct and 1.4 – 6.0%, respectively. The corresponding ranges for ACTB were 0.2 – 0.4 Ct and 0.7 – 1.6%.

Interfering Substances

Testing was performed to identify substances potentially found in plasma samples that could interfere with the proper functioning of the Epi proColon test and to determine potential sources of non-specificity of the assay. Interference was not observed when the substances were tested at the following concentrations: albumin (26 mg/mL), bilirubin (0.2 mg/mL), cholesterol (5 mg/mL), glucose (10 mg/mL), hemoglobin (10 mg/mL), triglycerides (12 mg/mL), K2EDTA (20 mg/mL), red blood cells (0.26% v/v), uric acid (0.235 mg/mL) and human sperm DNA (66 ng/mg). Positive results were detected when three substances were tested at higher concentrations: albumin (40 mg/mL), red blood cells (0.4% v/v) and human sperm DNA (100 ng/mL).

Blood and Plasma Handling

The amount of free circulating tumor DNA may be affected by specimen collection methods and sample storage and therefore requires a standardized blood collection and plasma preparation procedure. The instructions for the Epi proColon test define a stable procedure validated in a logistic study (Table 5-1). Whole blood specimens were collected from self-reported healthy subjects in BD Vacutainer K₂EDTA 10 ml tubes. The blood samples were spiked with CRC plasma immediately after blood draw (prior to plasma preparation). Spiked and un-spiked blood specimens were tested. Based on Ct-value measurement from a previously assayed aliquot of a CRC patient, the spike volume was calculated such that the projected Septin9 target concentration in a spiked specimen would be low enough to allow the observation of DNA decay during processing and storage, but high enough that observations would not be confounded with no or very low target in blood specimens.

There was no effect observed on the Epi proColon test result (qualitative and quantitative) for blood storage for an extended period of time, simulating delayed plasma processing in the laboratory (6 hours at room temperature) or overnight storage of whole blood in a refrigerator (24 hours at 2 to 8 °C).

There was no effect observed on the Epi proColon test result (qualitative and quantitative) of plasma being stored at temperatures different from -80°C, simulating shipment of plasma at cooled conditions (72 hours at 2 to 8 °C) or storage of plasma in a standard freezer (14 days at -25 to -15 °C).

There was no effect observed on the Epi proColon test result (qualitative and quantitative) of variations of the centrifugation procedures that simulate changes towards more gentle (e.g., single spin at lower speed + shorter time) or more harsh (double spin at higher speed + longer time) centrifugation conditions.

Table 5-1: Blood and Plasma handling Study

Handling Task	Outcomes
Standard Process	Standard process
Delay in plasma preparation	Extended storage (6 hours) of whole blood at room temperature does not impact outcome
Storage of plasma in standard refrigerator	Storage of plasma at 2 to 8°C for 72 hours does not impact outcome
Storage of plasma in standard freezer	Storage of plasma at -25 to -15°C for 14 days does not impact outcome
Single centrifugation, slow and short	Low speed, short time, single spin centrifugation does not impact outcome
Double centrifugation, fast and long	High speed, extended time, double spin centrifugation does not impact outcome
Overnight storage of whole blood	Overnight storage of whole blood at 2 to 8°C prior to plasma preparation does not impact outcome

6—Clinical Validation: Prospective Multicenter Pivotal Trial

Overview: Evaluation of the Epi proColon test was conducted with specimens from the prospective multicenter PRESEPT trial. For this trial, women and men ages 50-85, who were of average-risk for colorectal cancer, were enrolled at 32 clinical sites in the US and Germany. The clinical performance of the Epi proColon test was evaluated in a subset (1544) of the available 6,857 enrollees. All subjects with CRC and advanced adenomas, and a randomly selected subset of those having small polyps and those with no evidence of disease were included. The clinical performance was evaluated for sensitivity and specificity as determined by colonoscopy as the reference standard. The Epi proColon test was positive in 68% (30/44) of CRC patients and 21% (318/1500) of non-CRC individuals. Using calculations adjusted to the study population, the estimated clinical sensitivity for CRC was 68% (53 – 80%) at a clinical specificity of 80% (78 – 82%). The Epi proColon detected CRC at all stages and in both the distal and proximal colon and rectum.

The Pivotal trial design and results were presented to the FDA in a pre-PMA Meeting in Dec. 7th, 2011.

Pivotal Study Design:

- PRESEPT (“Prospective Evaluation of Septin9 Performance in CRC Screening”, NCT00855348) enrolled subjects from a CRC screening eligible population at average risk for CRC under the US Multi-Society Task Force Guideline (see inclusion/exclusion criteria below).
- Prior to bowel preparation for colonoscopy, blood was processed to plasma and archived at frozen conditions
- Plasma specimens from 6,857 subjects were available for inclusion into the Pivotal Study (see Figure 6-1).
- Data on subject age, gender, and ethnicity were recorded (see Table 6-2).
- The Epi proColon test performance was determined in archived plasma specimens from 1,544 subjects by comparison of the test result to clinical status determined by colonoscopy and pathological work-up.
- Clinical sensitivity for CRC was determined from Epi proColon test results obtained from all available CRC subjects (#CRC = 44).
- Clinical specificity for CRC was calculated from Epi proColon test results obtained from all available subjects with advanced adenomas (#AA = 621) and a random subset of subjects with small polyps (#SP = 435) or no evidence of disease (#NED = 444).
- Selection of SP and NED subjects was performed using a stratified random sampling allowing assessment of effects of demographic factors on test performance. Larger weight was put on strata of ethnic minorities and older age.

Subject Inclusions, Exclusions, Demographics, Subset Sampling

Inclusion and exclusion criteria were defined in the PRESEPT study protocol:

Patient Inclusion Criteria

- Capable of Informed Consent
- Capable of providing adequate health history
- Age 50 or older at time of colonoscopy (colorectal screening guideline eligible)
- Accessible for blood draw prior to start of bowel preparation for colonoscopy
- First colonoscopy in subject's lifetime

Patient Exclusion Criteria

- Anorectal bleeding or hematochezia within last 6 months for which patient sought medical attention
- Known iron deficiency anemia in the last 6 months for which patient sought or received medical attention
- Previous history of colorectal polyps or CRC
- High risk for colorectal cancer (2 or more 1^o relatives with CRC; 1 or more 1^o relative(s) < 50 years with CRC; known HNPCC or FAP)

Note: Subjects meeting eligibility, but failing inclusion or exclusion criteria were withdrawn from the study prior to blood draw.

Sample exclusion in the study was based on the following:

- Gross hemolysis (bright orange or red color)
- Protocol non-compliant collection, processing, storage or shipping
- Plasma samples with inadequate volume for Septin 9 analysis

Demographics and Subset Sampling

In the Pivotal study, a subset (1544) of the total of 6,857 subjects available from PRESEPT (see Figure 6-1) were tested. This included all CRC patients with a sufficient amount of plasma to estimate clinical sensitivity of the Epi proColon.

To estimate clinical specificity for CRC, all patients with advanced adenomas (AA) as well as a random stratified subset of subjects with small polyps (SP) and no evidence of disease (NED) from the PRESEPT cohort was selected as follows. The sampling strategy was designed to include, for SP and NED subjects each, an equal representation of gender, an age distribution aligning with the US census, and enhanced representation of ethnic minorities. Size of the subset was chosen such that precision of the specificity estimate was kept high (length of 95% confidence interval $\pm 2\%$ compared to $\pm 1\%$, if all 6,857 subjects would have been tested). At the

same time the stratification allowed assessment of the possible impact of demographic factors on Epi proColon test positivity.

Figure 6-1: Subject enrollment in the PRESEPT trial, and availability for the Pivotal Trial

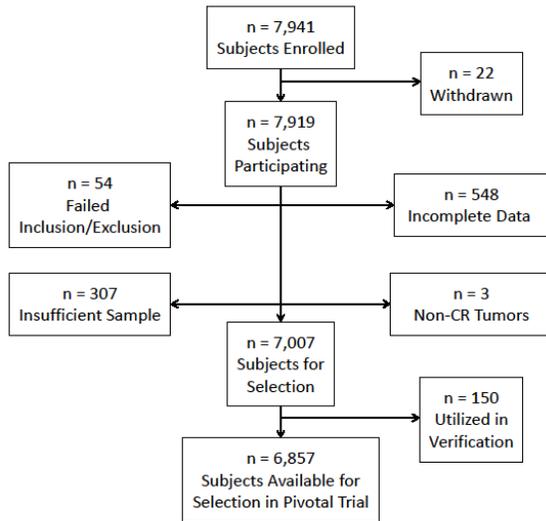


Table 6-2 provides information on the distribution across demographic factors found in the PRESEPT cohort (column 3) as well as the achieved distribution of specimens representing SP and NED subjects in sub-categories defined by demographic factors (Table 6-2 columns 4, 5). Deviations from stratification targets are due to the limited numbers of minority subjects above 69 years of age.

Table 6-2: Demographic Data for subjects enrolled in the PRESEPT and Results of Stratified Sampling

Factor	Value	PRESEPT Cohort N = 6,857	Pivotal Study #SP = 435	Pivotal Study #NED = 444
Gender	Male	45 %	49 %	50 %
	Female	55 %	51 %	50 %
Age	50 – 59	46 %	45 %	45 %
	60 – 69	42 %	30 %	29 %
	> 69	12 %	25 %	27 %
Race/Ethnicity	Caucasian	85 %	66 %	63 %

Factor	Value	PRESEPT Cohort N = 6,857	Pivotal Study #SP = 435	Pivotal Study #NED = 444
	African-American	10 %	21 %	25 %
	Others*	5 %	13 %	13 %
Country	U.S.A	75 %	84 %	84 %
	Germany	25 %	16 %	16 %

*Others include mainly Hispanic (3.6 %), but also American Indians, Alaska Natives, Asians, Native Hawaiians, Other Pacific Islanders, unclassified others.

Primary Endpoints: CRC Sensitivity and Specificity

The primary endpoints of the pivotal study were the sensitivity and specificity of the Epi proColon blood test as determined by comparison with the clinical classification based on the colonoscopy as the reference standard. The observed sensitivity for CRC was 68% (30 / 44).

The crude observed specificity for CRC was 79% (1182/1500). Adjusted specificity estimates were derived by respective weighting of diagnostic groups and demographic factors and bootstrapping. The adjusted specificity estimates based on the PRESEPT cohort was 80% (95% CI 78 – 82%).

Table 6-3: Clinical Sensitivity and Specificity for CRC in the Pivotal Trial. Adjusted specificity estimates weighted to the PRESEPT cohort and the US Census population.

Parameter	Point Estimate	CI 95%
Sensitivity	68.2 % (30/44)	53.4 – 80.0 %
Specificity	78.8 % (1182/1500)	76.7 – 80.8 %
Specificity (weighted to US census population)	79.1 %	77.0 – 81.4 %
Specificity (weighted to PRESEPT cohort)	80.0 %	77.9 – 82.1 %

Secondary Endpoints: Positivity by CRC and Non-Cancer Subgroups

The positivity of the Epi proColon test by tumor stage is displayed in Table 6-4. As indicated, detection rates increase by stage. When considering early stages, test detection in Stages I and II combined was 17/29 or 59% (95% CI 41 – 74%).

Table 6-4: Test positivity by CRC Stage

CRC Stage	Point Estimate	CI 95%
Stage I	41 % (7/17)	22 – 64 %
Stage II	83 % (10/12)	55 – 95 %
Stage III	80 % (8/10)	49 – 94 %
Stage IV	100 % (5/5)	57 – 100 %
Total Cancers	68 % (30/44)	53 – 80 %

The positivity of the Epi proColon test by tumor location is displayed in Table 6-5. Epi proColon detection rates of CRC are comparable for both sides of the colon.

Table 6-5: Test positivity by CRC Location

Location	Point Estimate	CI 95%
Proximal Colon	70 % (21/30)	52 – 83 %
Distal Colon	64 % (9/14)	39 – 84 %

The positivity of the Epi proColon test in non-CRC subgroups is displayed in Table 6-6. As indicated, the positive fraction in these groups is approximately 21%.

Table 6-6: Test Positivity by Non-CRC Subgroup

Clinical Group	Point Estimate	CI 95%
No Evidence of Disease (NED)	21.8 % (97/444)	18.3 – 25.9 %
Small Polyps	20.0 % (87/435)	16.5 – 24.0 %

Advanced Adenomas	21.6 % (134/621)	18.5 – 25.0 %
Total Non-CRC	21.2 % (318/1500)	19.2 – 23.3 %

Diagnostic Likelihood Ratio (DLR) and Predictive Values

Diagnostic likelihood ratios and predictive values were calculated based on the information provided by the PRESEPT cohort, i.e. using a bootstrap approach the specificity was weighted by age and ethnicity according to the PRESEPT cohort (Table 6-3) and the prevalence of CRC disease was estimated from the set of available 6,857 PRESEPT subjects. Point estimates together with 95% confidence intervals were calculated²⁴ and are provided in Table 6-7.

Table 6-7: Diagnostic Likelihood Ratios and Predictive Values²⁴

Parameter	Point Estimate	CI 95%
Positive Diagnostic Likelihood Ratio	3.41	2.72 – 4.27
Negative Diagnostic Likelihood Ratio	0.40	0.26 – 0.61
Positive Predictive Value	2.4 %	2.0 – 3.0 %
Negative Predictive Value	99.7 %	99.4 – 99.9 %

Additional analysis is presented in Appendix A1.

Influence of Demographic Variables on Test Performance

Our analysis of the impact of demographic variables on test performance is outlined in Appendix A-1. In non-CRC subjects elevated positivity was observed with age as well as in African American subjects. Positive and negative DLR values were calculated for available data, and predicted DLRs were calculated based on regression models to infer missing sub-classifications. In this analysis, though specificity was sensitive to age and ethnicity, the Epi proColon test was informative in all demographic categories, based on the concept that a test is 'informative' when DLR values are different from 1.

Summary of Results

- Clinical sensitivity for CRC was estimated as 68 % (95% CI, 53 – 80%)
- Adjusted clinical specificity for CRC was estimated as 80 % (95% CI, 78 – 82%)
- The Epi proColon test detects CRC at all stages
- The Epi proColon test is equally sensitive for CRC detection in the distal and proximal colon
- While specificity was sensitive to age and ethnicity, the Epi proColon test is informative in all demographic sub-categories where data was available

7—Clinical Validation: Prospective Multicenter Non-Inferiority Trial comparing Epi proColon and OC FIT-CHEK

Overview: In a prospective multicenter evaluation, the Epi proColon test was compared to a widely-used US commercial Fecal Immunochemical test (FIT). At 61 US clinical sites, asymptomatic women and men, ages 50-85, who were of average-risk for CRC were enrolled pre-colonoscopy. Additionally, persons with screen-detected colorectal cancer were evaluated at 10 days post-colonoscopy. All testing was conducted at a US independent clinical laboratory. The Epi proColon and FIT tests showed positivity in CRC of 73% (74/101) and 68% (66/97) and positivity in non-CRC of 18% (37/200) and 2% (5/193), respectively. By comparison of these results, the Epi proColon test was found to be statistically non-inferior to the FIT test with respect to sensitivity, but not specificity.

Study Design

In the second clinical validation trial (NCT01580540), the Epi proColon test was compared to the most widely-used US commercial FIT test (OC FIT-CHEK). This trial was recommended by the FDA to provide performance data for the Epi proColon test in comparison with immunochemical fecal occult blood testing. The rationale for this comparison was that FIT testing is the current approved non-invasive screening methodology, and warrants comparison.

The study was designed to collect matched blood and fecal specimens and clinical data from screening guideline-eligible subjects using colonoscopy as the reference method for detection of CRC. Subjects were recruited at 61 clinical sites in the US according to the following scheme:

- Subjects having CRC or a high suspicion of invasive CRC identified during screening colonoscopy were enrolled and provided blood and fecal samples at least 10 days after colonoscopy but prior to surgery or intervention
- Prospectively enrolled subjects provided blood and fecal samples prior to bowel prep for screening colonoscopy

Of 337 subjects enrolled in the study, 36 were excluded due to failure to meet inclusion/exclusion criteria. From the remaining 301 enrolled subjects, there were 101 CRC, 29 AA, 77 SP and 94 NED. Plasma samples were available from all 301 subjects. Fecal samples were not available from 11 subjects (4 CRC, 2 AA, 2 SP and 3 NED).

Both, the Epi proColon testing and OC FIT-CHEK testing were performed in a CLIA high complexity certified molecular diagnostics laboratory. Test outcome was compared to results obtained from colonoscopy for both methods.

Subject Inclusions, Exclusions, Demographics

- **Inclusions:** 301 women and men, ages 50-85, who were defined as average-risk with no family or personal history of CRC
- **Exclusions:** Individuals with a personal or family history of CRC or benign polyps in the colon or rectum, Crohn’s disease, inflammatory bowel disease, genetic syndromes including Lynch or FAP, and persons with anorectal bleeding or hematochezia or with known iron deficiency anemia

Table 7-1: detailed demographic data of the 301 study subjects.

		Colonoscopy Identified Cases	Prospectively Collected Cases				
Factor	Value	% of Total (n)	% of Total (n)	% (n)			
				CRC (n)	AA (n)	SP (n)	NED (n)
Gender	Female	32% (33)	62% (122)	0%	11% (14)	39% (47)	50% (61)
	Male	68% (69)	38% (77)	3% (2)	16% (12)	39% (30)	43% (33)
Age	50–59	24% (24)	64% (127)	1% (1)	13% (16)	39% (50)	47% (60)
	60–69	37% (38)	25% (49)	2% (1)	14% (7)	35% (17)	49% (24)
	> 69	39% (40)	11% (23)	0%	13% (3)	43% (10)	43% (10)
Race/ Ethnicity	Afr. American	11% (11)	14% (27)	0%	11% (3)	44% (12)	44% (12)
	Caucasian	69% (70)	70% (140)	1% (1)	15% (21)	38% (53)	44% (65)
	Hispanic	17% (17)	12% (24)	4% (1)	8% (2)	21% (5)	67% (16)
	Other	4% (4)	4% (8)	0%	0%	88% (7)	12% (1)
TOTAL		100% (102)	100% (199)	1% (2)	13% (26)	39% (77)	47% (94)

Primary Endpoint: Non-Inferiority for CRC Detection by Sensitivity and Specificity Analysis

Tables 7-2 to 7-4 present sensitivity and specificity results for the Epi proColon test and the FIT test.

The observed sensitivity for CRC on paired samples was 4.2% higher for the Epi proColon test. The 95% confidence interval (-16.2%; 8.1%) was strictly below the non-inferiority margin of 10% pre-set in the protocol. The sensitivity of the Epi proColon test is statistically non-inferior to the FIT test.

For specificity, the difference between tests was 16.6% in favor of the FIT with a 95% confidence limit (10.6%; 22.9%) around the estimate. This result does not demonstrate non-inferiority for specificity when compared to the non-inferiority margin of 20% pre-set in the protocol.

Table 7-2: Epi proColon Sensitivity and Specificity for all samples (n=301)

	Epi proColon Test	95% CI	
Sensitivity	73.3% (74/101)	63.9%	80.9%
Specificity	81.5% (163/200)	75.5%	86.3%

Table 7-3: Epi proColon Sensitivity and Specificity for paired samples (n=290)

	Epi proColon Test	95% CI	
Sensitivity	72.2% (70/97)	62.5%	80.1%
Specificity	80.8% (156/193)	74.7%	85.8%

Table 7-4: OC FIT-CHECK Sensitivity and Specificity for paired samples (n=290)

	FIT Test	95% CI	
Sensitivity	68.0% (66/97)	58.2%	76.5%
Specificity	97.4% (188/193)	94.1%	98.9%

A three-way comparison between the test results of the Epi proColon test and FIT with reference standard colonoscopy is presented in Table 7-5. 89% (86/97) of CRC patients were detected with at least one of the two tests.

Table 7-5: Three-way comparison of Epi proColon, FIT and Colonoscopy Results

Diagnostic Accuracy Criteria: Standard Colonoscopy						
Colorectal Cancer				Non-Colorectal Cancer AA, SP, NED		
	Epi proColon Positive	Epi proColon Negative	Total	Epi proColon Positive	Epi proColon Negative	Total
FIT Positive	50	16	66	1	4	5
FIT Negative	20	11	31	36	152	188
Total	70	27	97	37	156	193

Diagnostic Likelihood Ratio (DLR) and Predictive Values

Diagnostic likelihood ratios were estimated from the complete set of test results for Epi proColon and FIT. Predictive values were derived from the DLRs²⁴ using an estimated prevalence for CRC of 0.7%, but are provided for illustrative purpose only. The estimated negative likelihood ratios (and therefore the negative predictive values) are identical for both test methods. The estimated positive likelihood ratio (and therefore the positive predictive value) is higher for the FIT test.

Table 7-6: Diagnostic Likelihood Ratios and Predictive Values²⁴

Parameter	Epi proColon Test (n=301)		FIT Test (n=290)	
	Point Estimate	CI 95%	Point Estimate	CI 95%
Positive Diagnostic Likelihood Ratio	3.96	2.89 – 5.42	26.26	10.94 – 63.05
Negative Diagnostic Likelihood Ratio	0.33	0.24 – 0.46	0.33	0.25 – 0.44
Positive Predictive Value*	2.72%	2.00 – 3.68%	15.62%	7.16 – 30.77%
Negative Predictive Value*	99.77%	99.68 – 99.83%	99.77%	99.69 – 99.83%

*derived from the estimated DLRs using 0.7% as the estimate for prevalence for CRC.

Additional Results for CRC Detection

Both test methods detect CRC across all stages, different tumor locations and independent of demographic factors as shown in Tables 7-7 and 7-8. Additional analysis is outlined in Appendix A2.

Table 7-7: Positivity by Tumor Stage

CRC	Epi proColon Test		FIT Test	
	Point Estimate	CI 95%	Point Estimate	CI 95%
Stage 0	100% (2/2)	34.2 – 100%	0% (0/2)	0 – 65.8%
Stage I	61.5% (16/26)	42.5 – 77.6%	65.4% (17/26)	46.2 – 80.6%
Stage II	80.0% (16/20)	58.4 – 91.9%	80.0% (16/20)	58.4 – 91.9%
Stage III	65.2% (15/23)	44.9 – 81.2%	82.6% (19/23)	62.9 – 93.0%

	Epi proColon Test		FIT Test	
CRC	Point Estimate	CI 95%	Point Estimate	CI 95%
Stage IV	92.3% (12/13)	66.7 – 99.6%	58.3% (7/12)	32.0 – 80.7%
Unknown	76.5% (13/17)	52.7 – 90.4%	50.0% (7/14)	26.8 – 73.2%
Total	73.3% (74/101)	63.9 – 80.9%	68.0% (66/97)	58.2 – 76.5%

Table 7-8: Positivity by Tumor Location

	Epi proColon Test		FIT Test	
Location	Point Estimate	CI 95%	Point Estimate	CI 95%
Proximal Colon	73.1% (38/52)	59.7 – 83.2%	70.6% (36/51)	57.0 – 81.3%
Distal Colon	75.0% (27/36)	58.9 – 86.2%	69.4% (25/36)	55.1 – 82.0%

Results Summary

- The Epi proColon test was found to be statistically non-inferior to the FIT test with respect to sensitivity but not specificity.
- Based on DLR / NPV both tests performed equally well in confirming the absence of disease.
- Both tests identified similar numbers of CRC patients (though not necessarily the same individual)
- 88.7% of CRC patients were detected with at least one of the two tests
- Both tests support the primary objective of a screening modality—identification of patients with CRC at a curable stage. Both tests detected CRC at all stages
- Both tests were equally sensitive for CRC detection in the right and left colon

8—Precautions and Limitations (Instructions for Use)

Patient Precautions

- The Epi proColon test is an alternative screening method for patients who are defined as average risk for colorectal cancer by current screening guidelines, and who are unwilling, unable or do not undergo screening by other recommended screening methods.
- The Epi proColon test has not been evaluated in persons:
 - Considered to be at higher risk for developing colorectal cancer, or with a previous history of colorectal polyps or colorectal cancer. Persons at higher risk include those with a family history of colorectal cancer, particularly with two or more first-degree relatives with colorectal cancer, or one or more first degree relative(s) less than 50 years of age with colorectal cancer.
 - With known hereditary non-polyposis colorectal cancer (HNPCC) or familial adenomatous polyposis (FAP).
 - With anorectal bleeding, hematochezia, or with known iron deficiency anemia.
- Detection of colorectal cancer is dependent on the amount of free circulating tumor DNA in the specimen and may be affected by sample collection methods, sample storage, patient factors and tumor stage.
- There is insufficient evidence to report programmatic sensitivity of the Epi proColon test over an established period of time.
- CRC Screening guideline recommendations vary for persons over the age of 75. The decision to screen persons over the age of 75 should be made on an individualized basis in consultation with a healthcare provider.
- The Epi proColon test demonstrated non-inferiority to a FIT test (OC FIT-CHEK® Polymedco, Inc.), for sensitivity but not for specificity, indicating that the Epi proColon test exhibited a higher rate of false positive results compared to the FIT test. See Performance Characteristics in Section 13.
- Test results should be interpreted by a healthcare professional.

Laboratory Precautions Related to Real-Time PCR

- The Epi proColon test is for *in vitro* diagnostic use only
- This procedure is for professional laboratory use only and assumes familiarity with DNA extraction methods and real time PCR assays
- Compliance with good laboratory practices is essential to minimize the risk of cross-contamination between samples during and after the DNA extraction, bisulfite conversion, and purification procedure
- Use only single-use pipettes and filter tips to prevent cross-contamination of the patient sample
- Use of reference pipettes for pipetting extracted and bisulfite treated DNA is strongly recommended
- Good technique is important to prevent the introduction of nucleases into samples during the extraction procedure

- Do not freeze extracted DNA
- Epi proColon bisulfite solution is sensitive to oxygen contact; use only unopened tubes of Epi proColon Bisulfite Solution; do not store but discard any left-over solution
- When removing liquid from microtubes in multiple steps in the procedure, take care not to remove magnetic beads
- Strict separation of pre-PCR activities (e.g., plasma DNA extraction and purification, PCR setup) and post-PCR activities (e.g., real-time PCR) is highly recommended to prevent contamination by amplicons generated from previous PCR testing
- To prevent the release of any PCR product, used PCR plates should be placed in a resealable plastic bag immediately after removal from the PCR instrument, and the bag closed and disposed of in a dedicated PCR waste container
- Never open a used PCR plate or store a used PCR plate outside of the PCR instrument

Additional Precautions

- Do not mix kit components between kit lots
- Do not use kits or kit components beyond their stated expiration date
- Do not freeze whole blood K2EDTA blood or blood tubes
- The Epi proColon test kits do not contain infectious substances or agents that may cause disease in humans or animals
- All patient blood and plasma specimens should be handled as though they are capable of transmitting disease. Observe universal precautions and safe laboratory procedures as specified in the OSHA Standard on Bloodborne Pathogens, CLSI Document M29-A3, and any other appropriate biosafety practices as required by your laboratory.

Limitations

- This product has been validated for the combination of the Epi proColon Plasma Quick Kit (M5-02-001), the Epi proColon Sensitive PCR Kit (M5-02-002), and the Epi proColon Control Kit (M5-02-003) only. These kits and components (DNA extraction, bisulfite conversion or PCR kits) are not interchangeable or replaceable with other manufacturer's products.
- The Epi proColon test has been validated for use only with plasma derived from blood collected with BD Vacutainer® K₂ EDTA blood collection tubes (Becton Dickinson). Do not use this test with other clinical specimen types or with other blood collection tubes.
- The Epi proColon test has been validated for use only with the Applied Biosystems® 7500 Fast Dx Real-Time PCR Instruments with Sequence Detection Software v1.4 21 CFR Part 11 Module. Do not use with other instruments or software.
- Use of this test is limited to personnel experienced and trained in performing PCR assays. Good technique is essential and failure to follow instructions provided in these instructions may produce erroneous results.

9—Intended Market, Utilization, Support

Overview: This test is indicated for use in screening persons age 50 and older who are defined as average-risk for colorectal cancer by current screening guidelines. The market focus is the 23 million or 1 in 3 persons who currently are not participating in colorectal cancer screening by any recommended method.

The Company will provide training support and guides to all new laboratories adopting the test.

Market Demographic Focus

- Persons age 50 and over who are defined as average-risk for colorectal cancer by current screening guidelines
- The 1 in 3 persons who are non-adherent and do not participate in screening by current accordance with USPSTF recommended methods (fecal tests, colonoscopy and other imaging methods)

Testing Intervals

- Although longitudinal performance over time has not been established for the Epi proColon test, it is expected this would be similar to FIT and superior to stool guaiac test (gFOBT). Based on what is known about these comparators, annual testing is recommended.

Programmatic Screening

- There is insufficient evidence to report programmatic performance of the Epi proColon test over an established period of time.

Training and Support

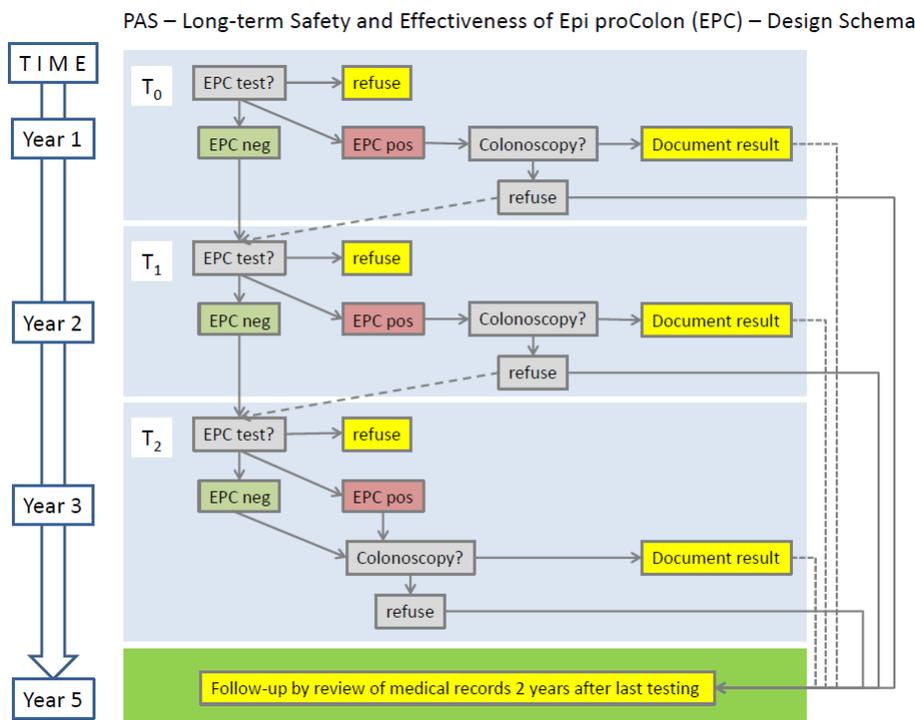
- Comprehensive training will be provided to all clinical laboratories purchasing the Epi proColon test kit
- Training materials will be developed to support the laboratory new test adoption process. These include but will not be limited to:
 - Epi proColon Instructions for Use
 - The Epi proColon QPC: The Quick Procedure Card: Training for the New User
 - The Epi proColon SPC: Blood Collection, Storage and Transport for Clinical Laboratory Draw Stations
 - The Epi proColon SPC: Blood Collection and Specimen Processing Guide for Clinical Laboratory Draw Stations
 - The Epi proColon Test Results Interpretation Guide
 - The Epi proColon Results Form
 - Procedure Tips Card

- Equipment and Materials Requirements Quick Reference Card
- Support materials for commercialization include the following brochures:
 - Physician brochure
 - Laboratory brochure
 - Patient brochure
 - Educational materials that include the Simple Truth CRC brochure, the Ready Reference and Selected Topics Educational Series of news bulletins
 - Customer Support and Website Quick Reference Card
- Support materials for new test adoption will be developed
 - Verification
 - Proficiency testing
 - Material Data Safety Sheets

10—Post Approval Study (PAS):

The post approval study proposal under consideration focused on determining the performance of the Epi proColon test when used over multiple years in a screening setting. The basic study scheme is outlined in the Figure 10-1.

Figure 10-1: Flow diagram outlining the proposed PAS study on programmatic performance of the Epi proColon test.



PAS Study Objective:

Determine the programmatic performance of Epi proColon (EPC) in a screening population of average risk for colorectal cancer (CRC) (consistent with proposed intended use population of the Epi proColon assay)

- Performance of EPC annual testing for 3 years evaluated according to the following:
 - Diagnostic yield: per round of testing & final
 - Test positivity, Positive Predictive Value (PPV): per round of testing & final
 - Programmatic sensitivity, Negative Predictive Value (NPV): final only
- Compliance to screening with EPC over time
- Adherence to diagnostic follow-up after positive EPC

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12 – Appendices

A1. Pivotal Study – Additional Data

Analysis By Demographic Factors

The impact of demographic variables on the performance of the Epi proColon test was tested with logistic regression methods. Based on the likelihood ratio test (significance level 0.05) “Age” and “Ethnicity” had an influence on the positive detection fraction (PDF). The factors “Country of origin” and “Gender” were not significant.

The test positive detection fractions (PDF) for sub-categories defined by diagnosis, age and ethnicity were calculated and are displayed in the Table A1-1. For CRC subjects, estimated PDFs are similar for all sub-categories with the exception of those four sub-categories not represented by a CRC patient (PDF = NA). For the non-CRC subjects the PDFs are slightly increased in African-American subjects, and there is a tendency towards increased PDF with increasing age.

Table A1-1: Results by Diagnosis, Age and Ethnicity

Ethnicity	Age Category	CRC			Non CRC		
		Positive	Negative	PDF	Positive	Negative	PDF
African-American	50-59	0	0	NA	33	94	26.0%
	60-69	2	1	66.7%	25	64	28.1%
	> 69	0	0	NA	12	30	28.6%
Caucasian	50-59	3	1	75.0%	56	363	13.4%
	60-69	13	6	68.4%	97	314	23.6%
	> 69	11	5	68.8%	68	195	25.9%
Other	50-59	0	0	NA	11	54	16.9%
	60-69	1	1	50.0%	8	44	15.4%
	> 69	0	0	NA	8	24	25.0%
Total	--	30	14	68.2%	318	1182	21.2%

Based on the statistical result that age and ethnicity significantly impact the PDF we investigated the extent to which the performance of the test varies for individuals of different demographic sub-groups. We used positive and negative diagnostic likelihood ratios (pDLR and nDLR) to describe the performance of the test for each demographic subgroup. The DLRs represent measures of the information contained in the test result and are easily obtained from the PDFs documented in Table A1-1. Table A1-2 provides observed DLRs together with 95% confidence intervals for the same demographic sub-groups as in Table A1-1 above. Note that for four sub-categories not represented by a CRC patient, the DLRs cannot be obtained

Table A1-2: Observed DLRs by Age and Ethnicity

Ethnicity	Age Category	Positive DLR		Negative DLR	
		Estimate	95% CI	Estimate	95% CI
African-American	50-59	NA	NA	NA	NA
	60-69	2.373	(0.998; 5.645)	0.464	(0.093; 2.309)
	> 69	NA	NA	NA	NA
Caucasian	50-59	5.612	(3.031; 10.391)	0.289	(0.053; 1.576)
	60-69	2.899	(2.040; 4.120)	0.413	(0.213; 0.803)
	> 69	2.659	(1.803; 3.922)	0.421	(0.203; 0.875)
Other	50-59	NA	NA	NA	NA
	60-69	3.250	(0.707; 14.941)	0.591	(0.147; 2.374)
	> 69	NA	NA	NA	NA
Total	--	3.216	(2.570; 4.024)	0.404	(0.262; 0.623)

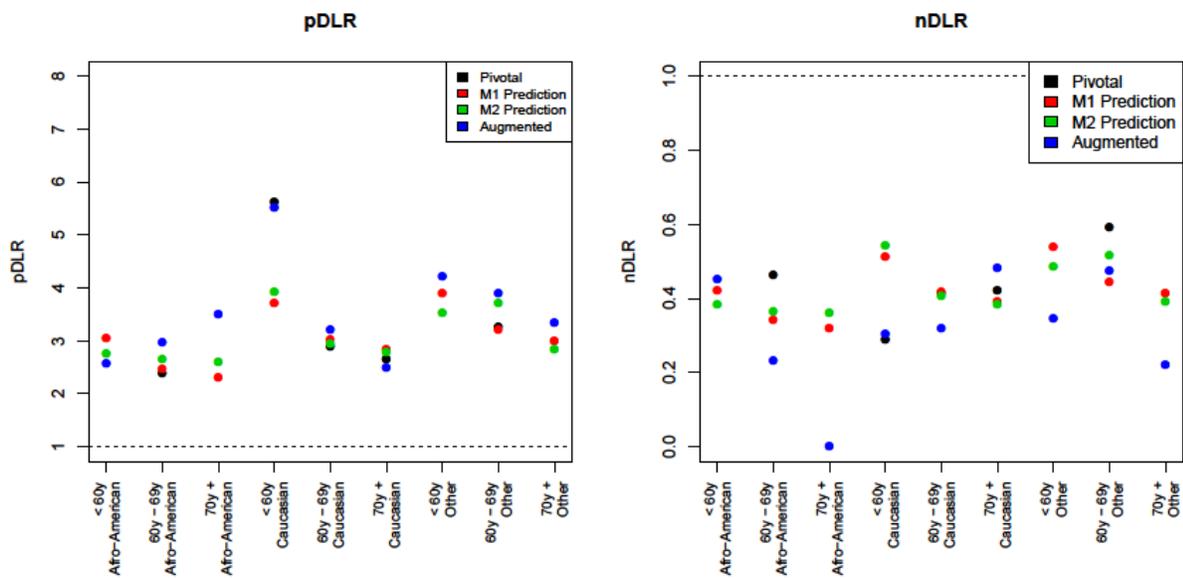
Two statistical models (logistic regression) were utilized to provide estimates (predictions) for the PDFs for all sub-categories. Both models (M1 and M2) include the statistical significant demographic factors “Age” and “Ethnicity” as predictors of the PDF. Model M2 is slightly more general as it allows for different effects of “Age” per “Ethnicity”-group. The model predictions were used to calculate positive and negative DLRs. Table A1-3 compares the observed DLRs from Table A1-2 with both sets of predictions. The predictions are close to the actually observed DLRs and therefore provide reasonable estimates for the sub-categories where direct observation was not possible. Based on this analysis, while specificity was sensitive to age and ethnicity, the Epi proColon test is informative in all demographic sub-categories where data was available.

Table A1-3: Observed and Predicted DLRs by Age and Ethnicity

Ethnicity	Age Category	Positive DLR			Negative DLR		
		Observed	M1 prediction	M2 prediction	Observed	M1 prediction	M2 prediction
African-American	50-59	NA	3.058	2.760	NA	0.422	0.382
	60-69	2.373	2.474	2.642	0.464	0.342	0.366
	> 69	NA	2.306	2.600	NA	0.319	0.360
Caucasian	50-59	5.612	3.707	3.916	0.289	0.512	0.542
	60-69	2.899	3.028	2.928	0.413	0.418	0.405
	> 69	2.659	2.822	2.779	0.421	0.390	0.385
Other	50-59	NA	3.901	3.518	NA	0.539	0.487
	60-69	3.250	3.204	3.719	0.591	0.443	0.515
	> 69	NA	2.988	2.826	NA	0.413	0.391

Figure A1-4 graphically displays the sets of DLRs documented in Table 6-9. In addition, results from a data augmentation method are presented exploiting data generated on 101 CRC patients from the FIT non-inferiority trial (see Section 7). Also these results are reasonably close to the observed DLRs and support the notion that the Epi proColon is informative for patients from all demographic subgroups. However, it is acknowledged that the augmentation with CRC data from a different study design may be criticized.

Figure A1-4: DLRs by Age and Ethnicity: Observed in Pivotal Study, Predicted by Model M1 and M2, Estimated after Augmentation with CRC data from FIT Non-inferiority Study. Positive (left panel) and negative DLR (right panel)



A2 – Additional Data – Non-Inferiority Trial

Table A2-1: Positivity by Demographic Factor Gender

Gender	Epi proColon Test		FIT Test	
	Point Estimate	CI 95%	Point Estimate	CI 95%
Female	78.8% (26/33)	62.2 – 89.3%	63.3% (19/30)	45.5 – 78.1%
Male	70.6% (48/68)	58.9 – 80.1%	70.1% (47/67)	58.3 – 79.8%

Table A2-2: Positivity by Demographic Factor Age

Age group (years)	Epi proColon Test		FIT Test	
	Point Estimate	CI 95%	Point Estimate	CI 95%
50 – 59	72.0% (18/25)	52.4 – 85.7%	75.0% (18/24)	55.1 – 88.0%
60 – 69	78.4% (29/37)	62.8 – 88.6%	69.4% (25/36)	53.1 – 82.0%
70 – 88	69.2% (27/39)	53.6 – 81.4%	62.2% (23/37)	46.1 – 75.9%

Table A2-3: Positivity by Demographic Factor Ethnicity

Ethnicity	Epi proColon Test		FIT Test	
	Point Estimate	CI 95%	Point Estimate	CI 95%
African-American	90.0% (9/10)	59.6 – 99.5%	70.0% (7/10)	39.7 – 89.2%
Caucasian	71.4% (50/70)	60.0 – 80.7%	73.5% (50/68)	62.0 – 82.6%
Others	71.4% (15/21)	50.0 – 86.2%	47.4% (9/19)	27.3 – 68.3%