Study Name: PREEMPT CRC: Prevention of Colorectal Cancer Through Multiomics Blood Testing

NCT Number: NCT04369053

Date: January 5, 2024

CLINICAL VALIDATION OF FREENOME'S METHYLATION BLOOD TEST FOR THE EARLY DETECTION OF COLORECTAL CANCER

STATISTICAL ANALYSIS PLAN

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List of Abbreviations and Definitions

Term	Definition
5PL	Five-Parameter Logistic
AA	Advanced Adenoma
ACN	Advanced Colorectal Neoplasia
ALL	All Enrolled Subject Population
cfDNA	Cell-Free DNA
CI	Confidence Intervals
CpG	A Cytosine Followed by A Guanine
CRC	Colorectal Cancer
CS	Colonoscopy
ctDNA	Circulating Tumor DNA
DMC	Data Monitoring Committee
EC	Extraction Control
Enrolled*	Enrolled* Subjects are defined as subjects enrolled on or after April 5th, 2021
FAS	Full Analysis Set
FMBT-CRC/FMBT-CRC-Methylation	Freenome's Blood-Based, Methylation Test
FN	False Negative
FP	False Positive
FRNM-MP	FMBT-CRC Multiomics Platform

Term	Definition
FRNM-SP	Freenome's proprietary cloud-based Software Platform
HMF	Hypermethylated Fragments
HP	Hyperplastic Polyps
IBD	Inflammatory Bowel Disease
Index Lesion	The most clinically significant lesion among synchronous lesions
IUP	Intended Use Population
IVD	In Vitro Diagnostic
meCpG	A methylated cytosine followed by a guanine
NAA	Non-Advanced Adenoma
NC	Negative Control
NEG	Negative; absence of confirmed CRC, Advanced Adenoma, and Non-Advanced Adenoma
NGS	Next Generation Sequencing
NIBSC	National Institute for Biological Standards And Control
NLR	Negative Likelihood Ratio
NPV	Negative Predictive Value
NTC	No Template Control
PC	Positive Control
PLR	Positive Likelihood Ratio
PPV	Positive Predictive Value

Term	Definition
SEER	Surveillance, Epidemiology, And End Results
Sn	Sensitivity
SOC	Standard of Care
SAS	Safety Analysis Set
Sp	Specificity
SSA/P	Sessile Serrated Adenoma/Polyp
TN	True Negative
TP	True Positive

Synopsis

Study Design

PREEMPT CRC was an observational, prospective, multi-center study including participation from over 130 different clinical sites widely distributed across the U.S., and one site outside the U.S., the Cleveland Clinic Abu Dhabi. This Institutional Review Board-approved study enrolled asymptomatic, average-risk subjects who met eligibility criteria and provided informed consent. The blood collection was done prior to undergoing bowel preparation for standard of care (SOC) screening colonoscopy (CS). Subjects had the option to have blood collection done either at the study site or via mobile phlebotomy services, in which case the subject could have blood collected in their home or other location of preference. Subjects had up to 50 mL of blood collected. De-identified blood specimens were sent to Freenome for processing, testing, and storage. Specimens will be tested by Freenome laboratory personnel blinded to the results of the SOC screening CS. Subjects underwent a SOC screening CS within 90 days after the blood collection.

Study Objectives

Primary Objectives

The co-primary objectives of this study are to (using CS with histopathology as the reference method):

- Estimate the sensitivity of the FMBT-CRC for colorectal adenocarcinoma (CRC)
- Estimate the specificity of the FMBT-CRC for subjects with no evidence of advanced colorectal neoplasia (ACN, comprising advanced adenoma and CRC)
- Estimate the negative predictive value of the FMBT-CRC for the detection of non-ACN subjects among those with negative FMBT-CRC results.
- Estimate the positive predictive value of the FMBT-CRC for the detection of ACN subjects among those with positive FMBT-CRC results.

Endpoints for all primary objectives must be met prior to evaluating the endpoint for the secondary objective.

Secondary Objective

- Estimate the sensitivity of the FMBT-CRC for advanced adenoma (AA).

Study Endpoints

Primary Endpoints

- Sensitivity of the FMBT-CRC for CRC
- Specificity of the FMBT-CRC for non-ACN
- Negative predictive value of the FMBT-CRC for non-ACN
- Positive predictive value of the FMBT-CRC for ACN

Secondary Endpoint

- Sensitivity of the FMBT-CRC for AA

Study Population

The intended use population (IUP) for this test is adults of either sex, 45 years or older, who are at average risk for CRC. The study enrolled a representative sample of the population of subjects who were at average risk for the development of CRC and underwent CRC SOC screening CS from sites across the U.S. and one site internationally. The typical subject came to a study site for other health concerns, and if they met eligibility criteria, were informed of the PREEMPT CRC study and asked if they would participate. Enrolled subjects ranged in age from 45-85.

1. Introduction

1.1. Background

CRC is the fourth most frequently diagnosed cancer and the second leading cause of cancer death in the United States. In 2022, an estimated 106,180 new cases of colon cancer and

44,850 new cases of rectal cancer were expected to occur in the United States, and it was estimated that 52,580 people would die from CRC (American Cancer Society, 2022). Screening for persons at average risk for CRC typically begins at age 50, and individuals at average risk have traditionally been defined as those aged ≥ 50 years without personal history of inflammatory bowel disease (IBD), adenomas, or CRC; without a family history of CRC or AA; and without symptoms such as rectal bleeding (U.S. Preventive Services Task Force, 2016; Rex et al., 2017; Provenzale et al., 2018). Registry data from the National Cancer Institute's (NCI) Surveillance, Epidemiology, and End Results (SEER) program indicated an increased incidence of CRC in African Americans prior to age 50 years (Howlader et al., 2019), which led to the recommendation in 2005 that CRC screening in African Americans begin earlier, at age 45 (Rex et al., 2017). More recently, epidemiologic reports suggest that the incidence of CRC may be increasing in adults aged < 50 years (Bailey et al., 2015; Meester et al., 2018; Peterse et al., 2018), leading the American Cancer Society in 2018 to recommend considering CRC screening in adults aged 45-49 years (Wolf et al., 2018). The U.S. Preventive Services Task Force also expanded the recommended ages for CRC screening to 45 to 75 years (Recommendation B) in 2021 (U.S. Preventive Services Task Force, 2021). Accordingly, private insurance plans have begun providing coverage for CRC screening in adults aged ≥ 45 years in 2022 (Fight CRC, 2022).

The U.S. Preventive Services Task Force recommended a 1-3 year screening interval for stool based tests that detect DNA biomarkers for cancer in cells shed from the lining of the colon and rectum into stool (sDNA-FIT) (U.S. Preventive Services Task Force, 2021). The FMBT-CRC detects DNA biomarkers for cancer and would likely receive a similar recommendation assuming appropriate sensitivity and specificity were established. Although the majority of colorectal polyps are benign and will never develop into colorectal cancer, it's generally accepted that the vast majority of colorectal cancer is caused by colorectal polyps. Colorectal polyps are defined as any discrete mass of tissue protruding into the lumen (opening inside the bowels) and are classified based on their cellular features (e.g., low/high grade dysplasia vs non-dysplastic), morphology (e.g., sessile vs pedunculated), and histology (e.g., serrated vs non-serrated), which in turn are related to their risk of malignancy (propensity to become cancer). Colorectal polyps are generally placed into two groups: (1) serrated lesions and polyps; and (2) conventional adenomas (Stryker et al., 1987; Lin et al., 2021). All conventional adenomas are dysplastic, and are also classified as tubular, villous, or tubulovillous. Per the central pathology review workflow, the serrated lesions and polyps group included hyperplastic polyps, which are not considered precancerous if they are diminutive or small (i.e. < 10mm diameter), sessile serrated polyps, and traditional serrated adenomas (Rex et al., 2017). For the purposes of the statistical analysis, and based on FDA feedback, hyperplastic polyps ≥ 1.0 cm

will be differentiated from the sessile serrated lesions group in a separate subcategory under non-advanced adenoma. The group of serrated lesions and polyps and conventional adenomas that are at greatest risk of malignancy are grouped as “advanced adenomas" as follows:

- Adenoma with carcinoma in situ or high-grade dysplasia, any size
- Adenoma, villous growth pattern (≥ 25%), any size
- Adenoma ≥ 1.0 cm in size
- Sessile serrated adenoma/polyp (SSA/P) with or without cytological dysplasia ≥ 1.0 cm (excludes hyperplastic polyps ≥ 1.0 cm)
- Traditional serrated adenoma (TSA), any size

Freenome has developed the FMBT-CRC for CRC screening that will enable improved adherence versus current CRC screening modalities, which remain either invasive or stool-based. In addition, use of the test is expected to improve the identification of clinically actionable disease, defined as disease (i.e., ACN) for which colonoscopic intervention has demonstrated health outcome benefit (Zauber et al., 2012; Corley et al., 2014). This in turn will improve the risk-benefit ratio for patients by increasing the likelihood that those patients directed by the test to undergo a diagnostic CS, with its attendant costs and complications, will benefit from it. The purpose of the current study is to validate FMBT-CRC performance and support regulatory approval.

2. Device Description

The FMBT-CRC is a qualitative next generation sequencing (NGS) *in vitro* diagnostic (IVD) test for the detection of methylation signal associated with ACN in plasma derived from whole blood specimens. The FMBT-CRC utilizes circulating cell-free DNA (cfDNA) from plasma of peripheral whole blood collected in [redacted] Cell-Free DNA BCT® blood collection tube (BCT). A positive test result may indicate the presence of CRC or AA or both.

The FMBT-CRC-Methylation test uses targeted next generation sequencing (NGS) to assess hypermethylated fragments (HMFs) across many genomic loci. These inputs are analyzed by an artificial intelligence and machine learning (AI/ML)-enabled classification model that combines the methylation values from all loci with a non-linear integrative function to generate a score and compare it to a threshold learned during model training, yielding a qualitative result. Scores at or above the threshold result in a positive call. Scores below the threshold result in a negative call. The overall test system is comprised of the following:

- FMBT Blood Collection Kit

- FMBT Pre-Analytic Subsystem
- FMBT-CRC Methylation Subsystem
- FRNM Software Platform

The FMBT-CRC methylation subsystem generates bioanalytical data derived from whole blood samples that are collected from patients by licensed healthcare providers using a collection kit provided by Freenome. The collection kit utilizes two BCTs. After whole blood samples are received at Freenome's clinical laboratory, plasma is isolated, then aliquoted and stored at -80℃ for future analytical processing appropriate for each respective assay. Raw data generated from the subsystem are input into the FMBT-CRC Software Platform (FRNM-SP) that includes pipeline software to process the raw sequencing data, the classification model which calculates the score, and test report generation software that outputs the final report (see Figure 1).

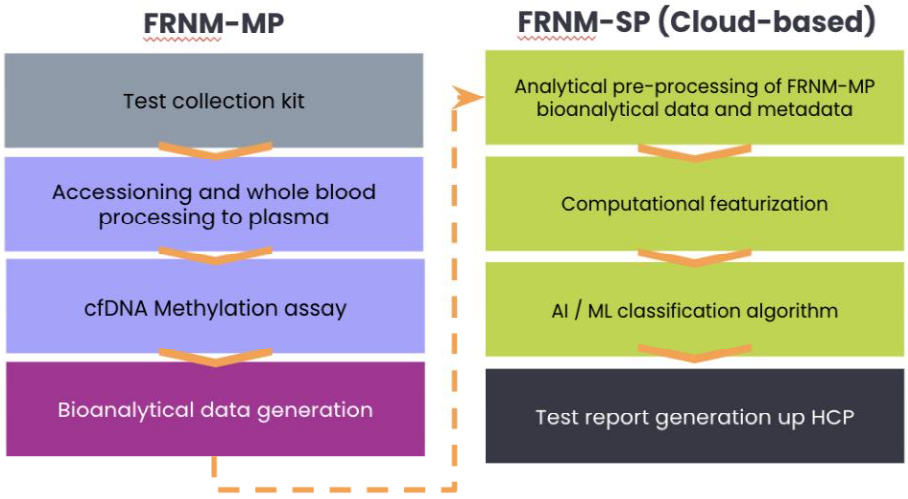

Figure 1 - FMBT-CRC Overall Test System

2.1. Methylation Subsystem

Dysregulation of DNA methylation patterns plays a critical role in tumorigenesis in many cancers (Kay et al., 1995). It is well established that tumors shed DNA into the blood via a variety of

active and passive processes (Tsang & Lo, 2006), and detection of aberrant methylation patterns among circulating tumor DNA (ctDNA) can be used to identify the presence of tumors non-invasively (Yamaguchi et al., 2003). [redacted] This panel was then used in case-control biomarker discovery studies conducted in-house with prospectively collected plasma samples from non-ACN (including non-advanced adenomas), AA and CRC patient groups. Through multiple rounds of statistical and machine learning classifier analysis, we identified a final panel that enables accurate classification of ACN from plasma-isolated cfDNA. Specifically, the methylation component of FMBT-CRC determines if a patient sample contains cfDNA methylated at CpG (a cytosine followed by a guanine) sites. [redacted] A fragment corresponds to a single cfDNA molecule in the source sample. [redacted]

[redacted]

[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

[redacted]	[redacted]	[redacted]
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[redacted]	[redacted]	[redacted]
[redacted]	[redacted]	[redacted]

2.2. Subsystem Controls

The methylation subsystem utilize a number of controls as part of the workflow. These controls are set forth below (Table 2).

Table 2 - Methylation Subsystem Controls

Control	Description
Positive Control (PC)	Sheared genomic DNA from a cell line with a known HMF rate similar to early-stage CRC samples.
Negative Control (NC)	Sheared genomic DNA from a cell line known to lack hypermethylation in CRC biomarker regions that is PCR amplified and size selected.
Extraction Control (EC)	A control used to assess cfDNA extraction success. The composition is identical to the PC.
No Template Control (NTC)	Non-template control or no-template control; an assay control that does not contain a nucleic acid template necessary to generate the assay product through an assay process; used to detect nucleic acid contamination in assay reagents, consumables, or instruments.

2.3. FRNM Software Platform (SP)

The molecular data from the laboratory processing of the blood samples are analyzed by the FRNM-SP software. FRNM-SP is proprietary cloud-based software that includes: Analytical pre-processing (e.g. sequence alignment and quality control) of input data derived from laboratory processing of the sample, computational "featurization," which transforms pre-processed inputs into the quantitative features required by a trained ML model, and application of the trained ML classification algorithm to the featurized inputs.

Freenome's computational pipeline and model is shown below in Figure 2. This pipeline launches when it receives a notice that the data from the sample processing is available. The methylation workflow inputs to FMBT-SP are a BCL file from an [redacted] sequencer, and a samplesheet from SLIMS that provides metadata about the BCL file.

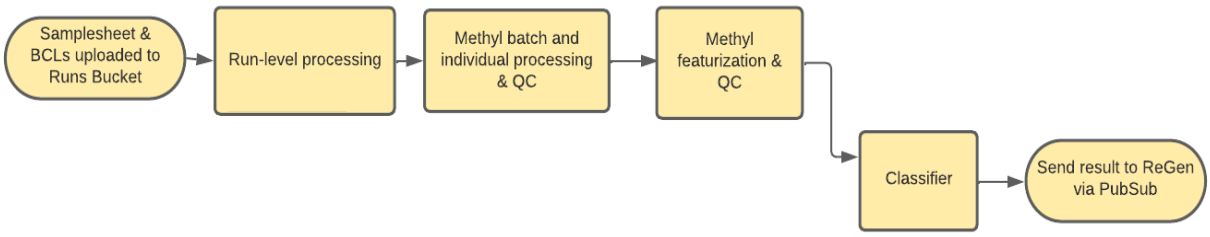

Figure 2 - Freenome FMBT-CRC-Methylation computational pipeline

The FMBT-CRC Methylation classifier workflow compares the methylation score directly to a methylation-specific threshold to determine the diagnostic result, positive or negative. This is shown in Figure 3.

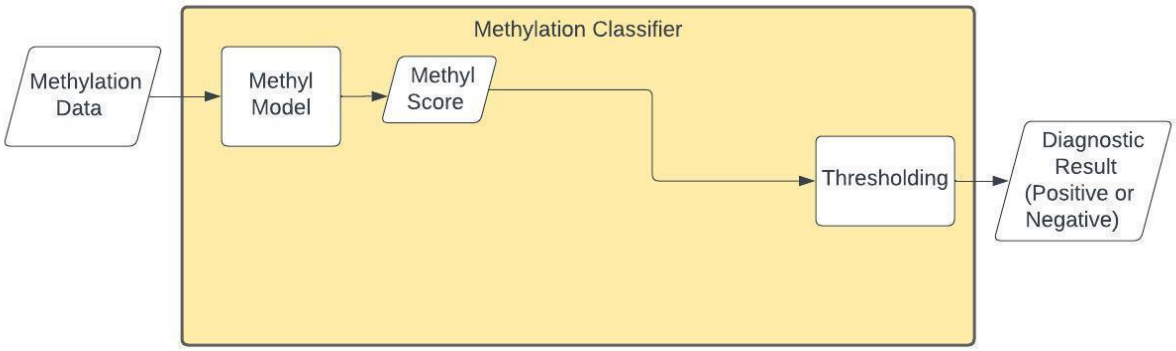

Figure 3 - Freenome FMBT-CRC-Methylation Classifier Workflow

3. FMBT-CRC Classification Model

3.1. Detection of ACN

As noted in Section 2.3, the FMBT-CRC-Methylation Classification Model generates a qualitative result by incorporating inputs from the methylation subsystem. These inputs are analyzed by an artificial intelligence and machine learning (AI/ML)-enabled classification model that combines the methylation values from all loci with a non-linear integrative function to generate a score and compare it to a threshold learned during model training, yielding a qualitative result. Scores at or above the threshold result in a positive call. Scores below the threshold result in a negative call.

3.2. CpG Methylation Score

The summarized value for methylation signal is based on counting HMFs identified in the cfDNA NGS data. The model first aligns fragment sequences to the human reference genome in order to associate each fragment with a predetermined genomic region. Genomic regions are further subdivided into sequential 100 base pair (bp) bins, to address the high degree of CpG density inhomogeneity along the genome. In model training, for each bin *i*, a large number of fragments derived from healthy donor samples are compared to fragments derived from ACN patient samples, and a per-bin HMF methylated CpG threshold (s_i) is learned. Fragments intersecting bin I and with a methylated CpG count at or above the s_i threshold are deemed to be

hypermethylated. Once the model has assessed all available fragments and identified any HMFs, it produces a total HMF count (*H*):

$$H = \sum_i \#\{\text{HMFs in bin } i\}.$$

Because the number of eligible fragments varies from sample to sample due to differences in cfDNA extraction and capture behavior, the model also computes the number of eligible fragments per bin: those intersecting the bin and with at least s_i CpGs (either methylated or unmethylated). The total eligible fragment (E) count is computed as:

$$E = \sum_i \#\{\text{eligible fragments in bin } i\}.$$

Finally, the HMF ratio is defined as $R = H/E$. The HMF ratio provides the single summarized value for CpG methylation signal.

3.3. Model ensemble

Because the FMBT-CRC-Methylation classification model, like most ML models, requires hyperparameter optimization, cross-validation was used in model training. Specifically, the full training data set was iteratively split into training, tuning, and test folds. (Tuning folds are used for hyperparameter selection, and test folds provide provisional model performance estimates to guide the development process.) [redacted]

3.4. Classification

To enable classification, the FMBT-CRC-Methylation ML classification model learned a classification threshold (t) based on behavior in negative samples during training and selected to yield a desired specificity. If $S \geq t$, the classifier returns a positive call.

4. Scope of the Analyses

This statistical analysis plan describes the methods to be used for clinical validation analysis of biological samples and data collected under Freenome Protocol FRNM-004, *PREEMPT CRC: Prevention of Colorectal Cancer Through Multiomics Blood Testing*, Version 3.0. This statistical analysis plan should be read in conjunction with the PREEMPT CRC study protocol, Version 3.0.

PREEMPT CRC was an observational, prospective, multi-center study including participation from over 130 different clinical sites widely distributed across the U.S., and one site outside the U.S., the Cleveland Clinic Abu Dhabi. This Institutional Review Board-approved study enrolled asymptomatic, average-risk subjects who met eligibility criteria and provided informed consent.

Ensuring diversity in the participating patient population was a priority throughout this study. As part of this effort, the Sponsor focused on engagement with sites that contributed to the ethnic/racial as well as socioeconomic diversity of the study population. These sites included the participation of the Morehouse School of Medicine in Atlanta, Georgia (which has since presented in collaboration with the Sponsor on some of their experiences (Mills et al, 2022; Mills et al., 2021)), the University of Chicago Institute for Population and Precision Health in Chicago, Illinois, the Cleveland Clinic Abu Dhabi in the United Arab Emirates, as well as sites comprehensively representing regions across the U.S.

PREEMPT CRC began enrollment in May 2020, two months after the start of the COVID-19 pandemic. As such, the impact of the pandemic was closely monitored during execution of the study by both the Sponsor and the independent Data Monitoring Committee (DMC). Several mitigations for the impact of COVID-19 were devised and integrated into study plans. These plans included the engagement of a virtual enrollment platform, Science 37, to increase outreach to potential subjects by extending and providing an alternative, virtual pathway for patient engagement. Subjects were also provided options to help facilitate participation in a remote setting, such as e-consenting and sample collection via mobile phlebotomy. These mitigations were implemented in a manner consistent with guidance provided by FDA (FDA, 2021).

Despite mitigations for COVID-19 as described above, during the early phase of the study, the pandemic had a profound impact on the healthcare system and the willingness of the general population to access health care for other than emergency conditions. This was especially true of patients with more advanced age and significant comorbidities who were understood to have higher risks of morbidity and mortality associated with COVID-19. The pandemic impact likely introduced shifts and biases in the set of individuals participating during this period which are unmeasurable and cannot be corrected for by statistical adjustment. As it is critical to maintain blinding to the diagnosis of CRC cases in this study, this issue will be addressed by pre-specifying an exclusion date coinciding with when the study was most impacted by COVID-19 (Fleming et al., 2020). By removing this earlier time interval, which has higher potential bias and confounders, the analysis population will better represent the IUP. The natural milestone of April 5, 2021 was used as the exclusion date, which corresponds to the execution of the protocol revision. The protocol revision helped further implement COVID-19 mitigations and the time period of the revision coincided with much of the U.S. opening up vaccination to all populations, thus easing restrictions on mobility and improved willingness to access healthcare (The American Journal of Managed Care, 2021). Therefore, enrollment on or after April 5, 2021 marked the beginning of a more representative population and will be used as the enrollment period for subjects included in this analysis, defined as the Enrolled* group.

The potential impacts that COVID-19 had on early study participation is supported by observed fluctuations in the epidemiological prevalence of CRC, which was originally assumed to be 0.5% during early study surveillance. The cause for these fluctuations is believed to reflect changes in both healthcare practice and patient behavior during the early months of the pandemic. At the kickoff of the PREEMPT DMC in July 2020, the DMC noted that the study might be impacted by the challenges for people to get access to CS due to the pandemic, resulting in underrepresentation of the breadth of comorbidities present in the IUP. Freenome continued to monitor screening rates throughout the study, and in order to assure blinding was maintained, developed a methodology to estimate CRC rates based on an approximate range of cases undergoing central pathology review at the time, a “bin” estimate, from which CRC rates could be periodically monitored. The assumed prevalence of CRC was amended in the protocol to 0.3% on April 5, 2021, which increased the enrollment target from 13,715 subjects to 25,000 subjects. As part of this amendment, the Sponsor also increased the number of study sites from 90 sites to approximately 120 sites, eventually reaching approximately 200 sites participating in the study. By the time of the next DMC meeting in July 2021, the Committee noted the study was progressing well and the rate of CRC was increasing. In March 2022, after assessing the latest metrics for the study, the DMC assessed that the necessary number of CRC cases had been reached and aligned on the decision to close enrollment for the study.

Another impact the pandemic had on the study was the extended storage of blood samples. The slower than expected initial enrollment and the increased enrollment target caused by the lower assumed CRC prevalence required the Sponsor to extend the enrollment period for the study. As a result, samples were stored considerably longer than originally planned. The PREEMPT CRC samples will begin processing around July 2023. Using the April 5, 2021 (inclusive) cutoff date for the analysis population reduces the real-time plasma stability data needed to 27 months. In order to include samples from date of first enrollment, in May 2020, real-time plasma stability data would be needed for 39 months.

The clinical validation of the FMBT-CRC will be based on PREEMPT CRC samples from participants enrolled on or after April 5, 2021, due to the fact that subjects enrolled prior to this date were less likely to represent the IUP and it would be unlikely that the unmeasured factors could be accounted for in analysis. This population, which enrolled after vaccines allowed improved access to healthcare, had a CRC incidence rate more in line with epidemiological predictions. Revised power calculations, using the revised enrollment window, show that the study is still overpowered for estimating the primary and secondary endpoints with a high degree of confidence.

4.1. Intended Use and Study Population

The IUP for this test is adults of either sex, 45 years or older, who are at average-risk for CRC. The study population were adults of either sex ages 45-85, referred by a healthcare professional for CRC screening. Typical subjects presented for medical intervention in a health care setting and met CRC screening criteria for a CS. High-risk individuals and individuals with gastrointestinal symptoms warranting diagnostic or surveillance CS were ineligible. Subjects were considered enrolled after a determination was made by the Investigator that the subject was eligible to participate according to the inclusion/exclusion criteria and understood and signed the informed consent form. No waivers of inclusion or exclusion criteria were granted by the Investigator in this study. Study sites consisted of mostly gastroenterology specialty practice and general health care sites.

4.2. Inclusion and Exclusion Criteria

Inclusion Criteria

For enrollment to this study, subjects met all the following criteria:

1. 45-85 years of age (inclusive) within 30 days of enrollment (target ≥ 70% of subjects to be 65-85).
2. Willing to undergo a SOC CS within 90 days of blood collection.
3. Considered by a physician or healthcare provider to be “average-risk” for CRC.
4. Able and willing to provide blood samples per protocol.
5. Able to comprehend and willing to sign and date the written informed consent document(s).

Exclusion Criteria

Subjects were excluded from enrolling into the study if any of the following were met:

1. Family history
   - 1.1. At least one (1) first-degree relative diagnosed with CRC before the age of 60 (Note: First degree relatives include parents, siblings, and offspring).
   - 1.2. At least two (2) first-degree relatives diagnosed with CRC at any age.
   - 1.3. Known hereditary gastrointestinal cancer syndrome including but limited to the following:
     - Hereditary non-polyposis CRC syndrome or Lynch Syndrome
     - Familial adenomatous polyposis
     - Cowden’s Syndrome
     - Juvenile Polyposis Syndrome
     - MUTYH-associated Polyposis
     - Peutz-Jeghers Syndrome
     - Serrated Polyposis Syndrome
2. Personal history
   - 2.1. CRC or colorectal adenoma
   - 2.2. History of malignancy (except for non-melanomatous skin cancer) at the time of enrollment, and for the 5 years preceding enrollment
   - 2.3. IBD, including chronic ulcerative colitis and Crohn’s disease
   - 2.4. Total colonic resection
   - 2.5. Cystic fibrosis
   - 2.6. CS in the 9 years preceding enrollment with the exception of an incomplete colonoscopy, or for a poor or inadequate bowel prep
   - 2.7. Sigmoidoscopy or CT colonography in the 4 years preceding enrollment
   - 2.8. Stool DNA testing in the 2 years preceding enrollment
   - 2.9. Fecal occult blood testing or fecal immunochemical testing in the 6 months preceding enrollment

2.10. Requiring an immediate or emergent colonoscopy for the investigation of symptoms
2.11. Solid organ or bone marrow transplantation
2.12. Blood product transfusion in the 120 days preceding enrollment
2.13. Any trauma or surgery requiring overnight inpatient hospitalization in the 30 days preceding enrollment

3. A medical condition which, in the opinion of the investigator, should preclude enrollment into the study.
4. Participated or currently participating in a clinical research study in which an experimental medication has been administered during the 60 days leading up to and including the date of providing informed consent or may be administered through the time of the CS.
5. Known to be pregnant.

4.3. Blood collection

After meeting eligibility and completing consent, subjects had the option to have blood collection completed either at the study site or via mobile phlebotomy services, in which case the subject could have blood collected in their home or at another location of preference. In cases where blood collection was not successful, a redraw was allowable as long as the subject had not yet completed colonoscopy.

The blood samples were received from the courier and inspected to ensure they were in acceptable condition. A sample was failed and rejected or placed on hold if the specific failure or hold criteria were met. Lab personnel then conducted sample accessioning in the Simple Laboratory Information Management System. The plasma and buffy coat were isolated from whole blood using the automated easyBlood and STAR system on the Hamilton Microlab in the Freenome Clinical Laboratory. The isolated plasma was aliquoted and stored at -80℃. The samples are maintained in freezer units located in the limited badge access clinical laboratory.

4.4. Colonoscopy

Subjects underwent a SOC bowel preparation followed by SOC screening colonoscopy within 90 days of blood collection (to ensure CRC status has not changed between blood draw and CS). Steps were taken to ensure quality of bowel preparation and colonoscopy were sufficient to evaluate the presence or absence of ACN. In cases where a non-ACN subject did not meet adequate quality colonoscopy, a repeat colonoscopy was allowable within 90 days of blood

collection. Due to the impact that COVID-19 had during this study, sites were occasionally given waivers for completing CS within 120 days after the blood collection. All subjects were required to provide a colonoscopy report, and additionally where applicable, histopathology reports, imaging reports, or operative notes on cancer staging. Although no long-term follow-up is planned, subjects may also be contacted by site personnel for up to 3 years following the completion of the study to gather additional information (e.g., any new cancer diagnosis).

A non-ACN subject who had a bowel preparation rated as less than fair (i.e. poor or inadequate), or an incomplete CS, was considered unevaluable and therefore will be excluded from analysis of all primary and secondary endpoints. An ACN subject who had a bowel preparation rated as less than fair or had an incomplete CS will be included in the analyses. Poor bowel preparation was defined as semisolid stool that could not be suctioned or washed away and < 90% of mucosa was seen. Inadequate bowel preparation was defined as needing repeat preparation for screening. A complete CS was defined as reaching the cecum (reaching the junction between the small and large intestine if the cecum has been resected) or reaching the neo-cecum. Cecal intubation was documented by photographic evidence and/or documentation of cecal intubation.

4.5. Data collection

All subjects were assessed for eligibility and consented. Blood specimens were collected prior to bowel preparation for screening CS. Baseline information (e.g., demographic, lifestyle and concomitant medication, etc.) was collected. All subjects also provided a CS report. Any positive CS required follow-up data collection, including but not limited to, the CS report, the associated histopathology report if any lesions were biopsied or removed, the surgical pathology report if any surgery was performed to remove lesions identified during the CS, and any other reports (e.g., imaging reports) required to determine the tumor-node-metastasis stage (based on the American Joint Committee on Cancer Staging Manual, 8th edition).

4.6. Central Pathology Review

Subjects will have their clinical outcome determined by using the best available evidence of their disease state. The most clinically significant lesion (clinical significance is defined in rank order in Table 3) among synchronous lesions is defined as the index lesion. All CS reports, as well as local histopathology reports when applicable, were reviewed by a central pathologist (Tier 1) to categorize the histopathological categories of the index lesion (i.e., CRC, AA, NAA, and NEG), as per histopathological categories defined in the protocol as well as in detailed work

instructions for pathology review (Table 3). Cases that were categorized as CRC or as certain subtypes of AA (AA2.1, AA2.2, AA2.4, and AA2.5), and a subset comprising 5% of all NEG4.1 (the first NEG4.1 from each site, then every 20th NEG4.1 per site after that), were additionally assessed as a Tier 2 independent central pathology review of tissue samples (i.e. H&E slides, fixed tissue blocks, or digital images). Any inconsistency between the site-reported Tier 1 assessments and the central pathologist Tier 2 assessment triggered an adjudication by a third independent central pathologist to break the tie (Tier 3).

Table 3 - Histopathological categories and definitions, including subtype definitions for AA, NAA, and NEG

Cat.	Definition for Central Pathology Review	Definition for Statistical Analysis
1	Colorectal cancer (CRC), all stages (I-IV)	Colorectal cancer (CRC), all stages (I-IV)
2	Advanced adenoma (AA), including: 2.1. Adenoma with carcinoma in situ or high-grade dysplasia, any size 2.2. Adenoma, villous growth pattern (≥ 25%), any size 2.3. Adenoma ≥ 1.0 cm in size 2.4. Sessile serrated adenoma/polyp (SSA/P) with or without cytological dysplasia ≥ 1.0 cm and hyperplastic polyps (HP) ≥ 1.0 cm. 2.5. Traditional serrated adenoma (TSA), any size	Advanced adenoma (AA), including: 2.1. Adenoma with carcinoma in situ or high-grade dysplasia, any size 2.2. Adenoma, villous growth pattern (≥ 25%), any size 2.3. Adenoma ≥ 1.0 cm in size 2.4. Sessile serrated adenoma/polyp (SSA/P) with or without cytological dysplasia ≥ 1.0 cm 2.5. Traditional serrated adenoma (TSA), any size
3	Non-advanced adenoma (NAA)	Non-advanced adenoma (NAA)

Cat.	Definition for Central Pathology Review	Definition for Statistical Analysis
	3.1. ≥ 5 adenomas, all < 1.0 cm in size, non-advanced 3.2. 1 to <5 adenomas, all < 1.0 cm in size, non-advanced	3.1. ≥ 5 adenomas, all < 1.0 cm in size, non-advanced 3.2. 1 to <5 adenomas, all < 1.0 cm in size, non-advanced 3.3. HP ≥ 1.0 cm
4	Negative (NEG) 4.1. All SSA/P < 1.0 cm, and HP < 1.0 cm not in sigmoid or rectum 4.2. HP < 1.0 cm in the sigmoid or rectum 4.3. Negative upon histopathological review 4.4. No findings on colonoscopy, no histopathological review	Negative (NEG) 4.1. All SSA/P < 1.0 cm, and HP < 1.0 cm not in sigmoid or rectum 4.2. HP < 1.0 cm in the sigmoid or rectum 4.3. Negative upon histopathological review 4.4. No findings on colonoscopy, no histopathological review

4.7. Subject Disposition

The disposition of the subjects in this study is illustrated in the flowchart below (Figure 4), indicating the numbers reaching each stage and those excluded from the full analysis set in the study at that stage with a summary count for each of the major reasons for not proceeding. Sponsor is defining “all enrolled subjects” as subjects enrolled in the study. Sponsor is defining “enrolled*” subjects as those enrolled on or after April 5, 2021, and only those subjects will be included in the analysis set.

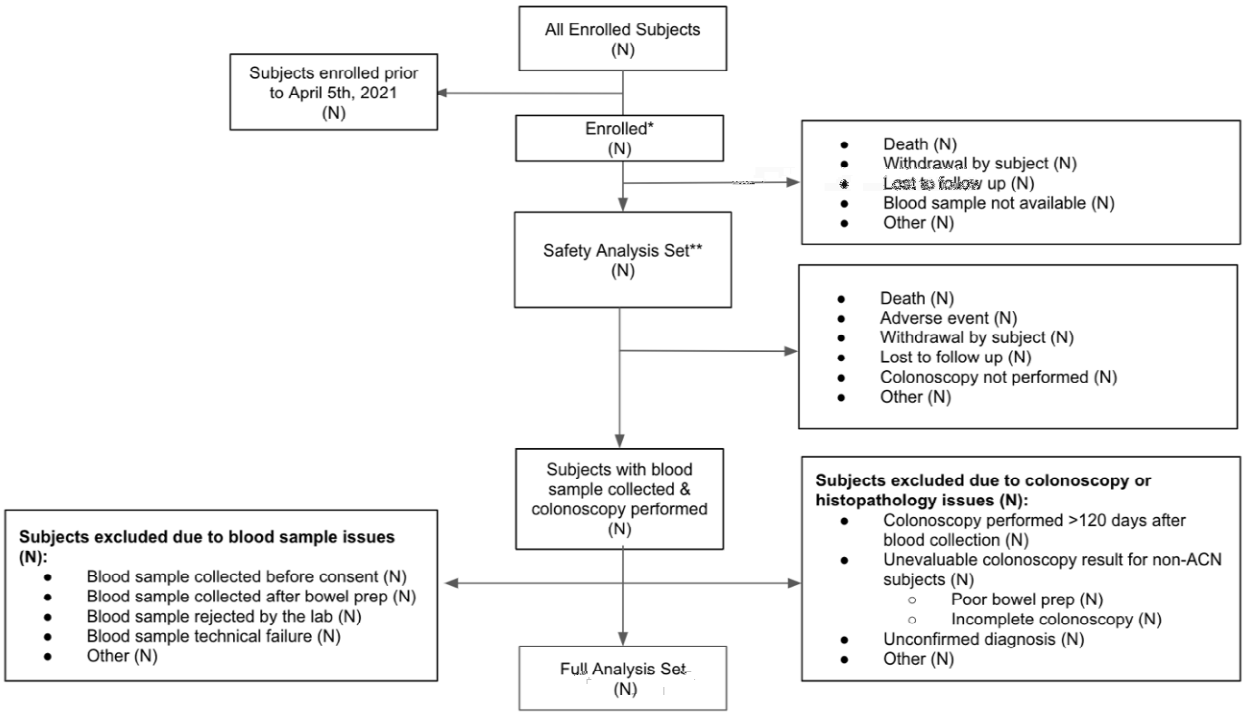

Figure 4 - Subject Disposition Flowchart

A subject may be discontinued and therefore excluded from all primary and secondary analyses due to the following reasons:

- Withdrawal by subject
- Physician decision
- Subject or Trial site terminated by Sponsor
- Adverse event
- Death
- Protocol deviation (refer to next section)

- Blood sample failure (e.g., resulting from errors in labeling, improper collection or handling, poor specimen quality, assay-related failures)
- Unevaluable patient due to CS (e.g., patient with only unevaluable colonoscopies, defined as incomplete CS or bowel preparation quality rated as poor or inadequate (unless diagnosed with CRC or AA))
- Other

4.8. Protocol Deviations

Protocol deviations and actions taken to resolve the deviations were noted on the designated source documents at each clinical site and described in study monitoring reports. Determination of whether a protocol deviation results in a subject being ineligible for final analysis will be reviewed on a case-by-case basis. All protocol deviations will be described in the final study report from the following categories.

- Consent related
- Inclusion/Exclusion Criteria related
- Blood Draw related
- Colonoscopy related

4.9. Blinding

The PREEMPT CRC study is a blinded study and follows blinding as per the study blinding plans. For this study, blinding is defined as preventing knowledge of subject-specific histopathological classifications or blood test results that could bias the study. Blood specimens were de-identified prior to being sent to Freenome for processing and storage. Specimen testing will be performed by laboratory personnel blinded to the results of the SOC screening colonoscopy. Subjects, their health care providers, and investigators will remain blinded to the results of FMBT-CRC.

To minimize risk of unintended unblinding at the time of data analysis, the study also maintained an unblinding plan which outline the Sponsor-level processes for unblinding specific roles/data, such as data preparation, data quality and control, and data analyses, as needed through

completion of this study (PL-00071). Unblinded roles are limited to a need-basis. Clinical Data Manager(s), Clinical Scientist(s), and Biostatisticians(s) can be unblinded if their defined responsibilities need to be conducted. Individuals granted access to unblinded data will not communicate results or characteristics of the unblinded data in any way that would expose information on subject-specific histopathological classifications (or any other blinded content) to individuals not granted access. Individuals granted access to the blinded data must not work on the classifier algorithms or have access to results of the blood test prior to study database lock. Individuals granted access to blinded data will be documented. The Sponsor will decide on the level of unblinding of PREEMPT CRC blinded data at the end of study.

4.10. Data Monitoring Committee

The DMC oversaw the PREEMPT CRC study and was responsible for:

- Determining the required number of events (e.g., evaluable subjects diagnosed with CRC) had been met to provide stopping guidance to Freenome.
- Monitoring data related to the quality of colonoscopies (specifically adenoma detection rate by gender, bowel preparation quality, and cecal intubation rate) to alert Freenome to sites or phlebotomists requiring remediation.
- Monitoring serious adverse events related to the study procedure (i.e., venipuncture for blood collection).

After the review of each Data Report was completed, the DMC Chairperson provided the official DMC recommendation to Freenome regarding the appropriateness of continuing the study, as well as any other recommendations relevant to study conduct and/or patient safety.

5. Analysis Populations

5.1. All Enrolled Subjects (ALL)

This set will include those subjects who enrolled (met eligibility criteria and consented).

5.2. Enrolled* Subjects (EN)

This set will include those subjects who enrolled on or after April 5, 2021, who met eligibility criteria and consented.

5.3. Full Analysis Set (FAS)

This set will include those subjects enrolled on or after April 5, 2021, who met eligibility criteria, consented, and have an evaluable Freenome test result, with confirmed diagnosis from the colonoscopy and best available evidence of histopathology, including central pathology review.

This will be the population used in the analyses of the primary and other endpoints.

5.4. Safety Analysis Set (SAS)

The safety analysis set is defined as enrolled* subjects with a study intervention. The intervention in this study was the attempted blood sample collection. All subjects in the SAS will be evaluated for Adverse Events, and have their demographics characterized in the analysis.

6. Study Objectives and Endpoints

6.1. Diagnostic Accuracy Estimation and Notation

All endpoints are based on groups of disease-positive and disease-negative subjects, with group membership varying as required by the endpoint definitions. Subject disease status is defined in terms of the four Categories listed in Table 3, or in certain cases, in terms of specific Category subtypes. Typical disease status definitions are listed in Table 4. Unless otherwise noted, the negative class for purposes of specificity, negative predictive value and positive predictive value estimation is the combination of Categories 3 and 4.

Table 4 - Disease positive/negative subject groups used in various sensitivity/specificity calculations related to endpoints

Sensitivity endpoint	Specificity endpoint	Disease positive	Disease negative

Sensitivity for CRC	Specificity for non-ACN	Category 1	Categories 3-4
Sensitivity for ACN	Specificity for non-ACN	Categories 1-2	Categories 3-4
Sensitivity for AA	Specificity for non-ACN	Category 2	Categories 3-4
Sensitivity for NAA	Specificity for NEG	Category 3	Category 4
N/A	Specificity for no CS findings	N/A	NEG4.4

Diagnostic accuracy data will be presented in the form of 2 x 2 tables, as shown in Table 5, which will be presented separately for each relevant endpoint.

Table 5 - Contingency table for the FMBT-CRC

FMBT-CRC	Clinical Outcome (CS with Histopathology)	
	Disease Positive	**Disease Negative**
Test Positive	TP	FP
Test Negative	FN	TN

- Table 5 includes the number of true positives (TP, those who have the disease and test positive), false positives (FP, those who do not have the disease but test positive), false negatives (FN, those have the disease but test negative) and true negatives (TN, those who do not have the disease and test negative).
- From the 2 x 2 contingency table, specificity and sensitivity will be assessed and reported with two-sided exact 95% confidence intervals (Wilson score method).
- Additional performance characteristics will also be presented, e.g., positive and negative predictive values; positive and negative likelihood ratios.

Some of the endpoints estimated as part of this study identify subjects in more detail than the 2 x 2 contingency table presented in Table 5, as shown in Table 6.

Table 6 - Detailed contingency table for the FMBT-CRC

FMBT-CRC	Clinical Outcome (CS with Histopathology)		
	CRC Positive	**AA Positive**	**Non-ACN Negative**
Test Positive	TP_{CRC}	TP_{AA}	$FP_{non\text{-}ACN}$
Test Negative	FN_{CRC}	FN_{AA}	$TN_{non\text{-}ACN}$

This study depends on standard performance characteristics for a screening test. The following endpoints will be assessed (primary and secondary endpoints in bold):

- The sensitivity (Sn) or TP rate of the test is defined as TP/(TP + FN). This will be presented for CRC, AA and ACN groups of subjects as follows:
  - **$Sn_{CRC} = TP_{CRC}/(TP_{CRC} + FN_{CRC})$**
  - **$Sn_{AA} = TP_{AA}/(TP_{AA} + FN_{AA})$**
  - $Sn_{ACN} = TP_{ACN}/(TP_{ACN} + FN_{ACN})$
- The specificity (Sp) or TN rate of the test is defined as TN/(TN + FP). This will be presented for the non-ACN, NEG, NEG 4.1 through 4.3, and NEG 4.4 (no CS findings) groups of subjects as follows:
  - **$Sp_{non\text{-}ACN} = TN_{non\text{-}ACN}/(TN_{non\text{-}ACN} + FP_{non\text{-}ACN})$**
  - $Sp_{NEG} = TN_{NEG}/(TN_{NEG} + FP_{NEG})$
  - $Sp_{NEG4.1\text{-}4.3} = TN_{NEG4.1\text{-}4.3}/(TN_{NEG4.1\text{-}4.3} + FP_{NEG4.1\text{-}4.3})$
  - $Sp_{NEG4.4} = TN_{NEG4.4}/(TN_{NEG4.4} + FP_{NEG4.4})$
- FP rate will be calculated for each specificity as 1-Sp.
- The positive predictive value (PPV) of the test is defined as TP/(TP + FP). This will be presented with respect to CRC, AA and ACN disease-positive definitions.
  - **$PPV_{ACN} = TP_{ACN}/(TP_{ACN} + FP_{non\text{-}ACN}) = (PLR_{ACN} * Prev_{ACN})/(1 + Prev_{ACN} * (PLR_{ACN} - 1))$**

- $PPV_{CRC} = TP_{CRC}/(TP_{ACN} + FP_{non\text{-}ACN}) = (PLR_{CRC} * Prev_{CRC})/(1 + Prev_{CRC} * (PLR_{CRC} - 1))$
- $PPV_{AA} = TP_{AA}/(TP_{ACN} + FP_{non\text{-}ACN}) = (PLR_{AA} * Prev_{AA})/(1 + Prev_{AA} * (PLR_{AA} - 1))$

- The negative predictive value (NPV) of the test is defined as TN/(TN + FN). This will be presented with respect to the non-ACN disease-negative definition.
  - **$NPV_{non\text{-}ACN} = TN_{non\text{-}ACN} /(TN_{non\text{-}ACN} + FN_{ACN}) = (1 - Prev_{ACN})/(1 + Prev_{ACN} * (NLR_{ACN} - 1))$**
- The positive likelihood ratio (PLR) is defined as:
  - $PLR_{ACN} = Sn_{ACN}/(1\text{-}Sp_{non\text{-}ACN})$
  - $PLR_{CRC} = Sn_{CRC}/[(TP_{AA}+FP_{non\text{-}ACN})/(N_{AA}+N_{non\text{-}ACN})]$
  - $PLR_{AA} = Sn_{AA}/[(TP_{CRC}+FP_{non\text{-}ACN})/(N_{CRC}+N_{non\text{-}ACN})]$
- The negative likelihood ratio (NLR) is defined as (1-Sn)/Sp.
  - $NLR_{ACN} = (1\text{-}Sn_{ACN})/Sp_{non\text{-}ACN}$
  - $NLR_{CRC} = (1\text{-}Sn_{CRC})/[(FN_{AA}+TN_{non\text{-}ACN})/(N_{AA}+N_{non\text{-}ACN})]$
  - $NLR_{AA} = (1\text{-}Sn_{AA})/[FN_{CRC}+TN_{non\text{-}ACN})/(N_{CRC}+N_{non\text{-}ACN})]$

For Sensitivity and Specificity, confidence intervals (CI) will be calculated using the Wilson score method. For PLR and NLR, the CI for the ratio of two independent proportions will be calculated using the non-iterative Score approach described by Nam (1995) [FDA, 2017]. For PPV and NPV, the CI will be calculated using the CI of the corresponding predictive value (PLR and NLR, respectively), where prevalence is a constant (FDA, 2017).

6.2. Study Objectives

Primary Objectives

The co-primary objectives of this study are to (using screening CS with histopathology as the reference method):

- Estimate the sensitivity for CRC (Sn_{CRC})

- Estimate the specificity for subjects with no evidence of advanced colorectal neoplasia ($Sp_{non\text{-}ACN}$)
- Estimate the NPV of the FMBT-CRC for the detection of non-ACN subjects among those with negative FMBT-CRC results ($NPV_{non\text{-}ACN}$).
- Estimate the PPV of the FMBT-CRC for the detection of ACN subjects among those with positive FMBT-CRC results (PPV_{ACN})

CI endpoints for all primary objectives must be met to declare the study a success.

Secondary Objective

The secondary objective will be evaluated individually, only if the co-primary objectives have been met.

- Estimate the sensitivity of the FMBT-CRC for AA (Sn_{AA})

6.3. Study Acceptance Criteria

Primary-Objective Acceptance Criteria

The primary objective of this study is to determine that FMBT-CRC is suitable for clinical use. The primary objective will be evaluated using co-primary endpoints of Sn_{CRC}, $Sp_{non\text{-}ACN}$, $NPV_{non\text{-}ACN,}$ and PPV_{ACN} computed from the FAS data.

Acceptance criterion for CRC sensitivity

- the lower bound of the 95% confidence interval for CRC sensitivity, calculated with Wilson's method, shall be greater than 65.0%

Acceptance criterion for non-ACN specificity

- the lower bound of the 95% confidence interval for non-ACN specificity, calculated with Wilson's method, shall be greater than 85.0%

Acceptance criterion for non-ACN NPV

- the lower bound of the 95% confidence interval for $NPV_{non\text{-}ACN}$ – $Prevalence_{non\text{-}ACN}$ shall be greater than 0.16%, where $NPV_{non\text{-}ACN}$ is calculated with NLR and the observed prevalence (FDA, 2017)

Acceptance criterion for ACN PPV

- the lower bound of the 95% confidence interval for PPV_{ACN} – $Prevalence_{ACN}$ shall be greater than 1.17%, where PPV_{ACN} is calculated with PLR and the observed prevalence (FDA 2017)

For the study to be a success, the acceptance criteria for Sn_{CRC}, $Sp_{non\text{-}ACN}$, $NPV_{non\text{-}ACN}$ and PPV_{ACN} must all be met.

Secondary-Objective Acceptance criterion

The secondary objective will be evaluated only if all the primary objectives are met. The acceptance criteria must be met to declare the success of the secondary objective.

- The lower bound of the 95% confidence interval for AA sensitivity calculated with Wilson's method shall be greater than 11.5%

6.4. Study Endpoints

Primary Endpoints

Sensitivity of FMBT-CRC for CRC

The co-primary endpoint for CRC sensitivity is a two-sided 95% CI based on Sn_{CRC}. The CI will be constructed using the Wilson score method and all FAS subjects identified as CRC by CS and best available evidence, including central histopathology. A lower CI bound that exceeds 65.0% corresponds to rejecting the null hypothesis at the one-sided α = 0.025 level.

Specificity of FMBT-CRC for non-ACN

The co-primary endpoint for non-ACN specificity is a two-sided 95% CI based on $Sp_{non\text{-}ACN}$. The CI will be constructed using the Wilson score method and all FAS subjects identified as non-ACN by CS and best available evidence, including central histopathology. A lower CI bound that exceeds 85.0% corresponds to rejecting the null hypothesis at the one-sided α = 0.025 level.

Negative predictive value of FMBT-CRC for non-ACN

The co-primary endpoint for non-ACN NPV is a two-sided 95% CI based on $NPV_{non\text{-}ACN}$ - $Prevalence_{non\text{-}ACN}$ The CI will be constructed using NLR and the observed non-ACN prevalence (FDA, 2017), all FAS subjects with a negative FMBT-CRC result, and disease status from CS and best available evidence, including central histopathology. A lower CI bound that exceeds 0.16% among FAS subjects corresponds to rejecting the null hypothesis at the one-sided α = 0.025 level.

Positive predictive value of FMBT-CRC for ACN

The co-primary endpoint for ACN PPV is a two-sided 95% CI based on PPV_{ACN} – $Prevalence_{ACN}$. The CI will be constructed using the PLR and observed ACN prevalence (FDA, 2017), all FAS subjects with a positive FMBT-CRC result, and disease status from CS and best available evidence, including central histopathology. A lower CI bound that exceeds 1.17% among FAS subjects corresponds to rejecting the null hypothesis at the one-sided α = 0.025 level.

Secondary Endpoint

Sensitivity of FMBT-CRC for AA

The secondary endpoint for AA sensitivity is a two-sided 95% CI based on the observed sensitivity point estimate (Sn_{AA}). The CI will be constructed using the Wilson score method and all FAS subjects identified as AA by CS and best available evidence, including central histopathology. A lower CI bound that exceeds 11.5% corresponds to rejecting the null hypothesis at the one-sided α = 0.025 level.

7. Sample Size Justification

The study should include a minimum of 56 evaluable CRC cases meeting the requirements for inclusion in the FAS analysis set for the primary sensitivity endpoint. Assuming a prevalence of 0.30% CRC cases, the minimum number required in the FAS is 56/0.30% = 19,000 subjects. Allowing for a 20% unevaluable rate, this gives a required enrollment of 24,000 subjects.

All of the sample size calculations assume the following:

- Power = 90%
- α = 0.025

- One-tailed test for greater than the lower bound.
- Statistical power for $Specificity_{non\text{-}ACN}$, $Sensitivity_{AA}$ and $Sensitivity_{CRC}$ were conservatively estimated assuming an exact-binomial distribution, which has greater coverage than Wilson score. Sample size was set such that 90% power was consistently achieved (Chernick 2012).
- The critical value for a given sample size was determined using the Wilson score method.
- From a meta-analysis, the prevalence of CRC is estimated to follow a beta distribution with parameters 3.264 and 816.77; the prevalence of AA is estimated to follow a beta distribution with parameters 34.265, 436.699.
- Given the critical values for $Sp_{non\text{-}ACN}$, Sn_{AA}, and Sn_{CRC}, in addition to the prevalence of AA, CRC, and non-ACN and estimated study size, a distribution of $NPV_{non\text{-}ACN}$ and PPV_{ACN} were generated, from which the 2.5th percentile was used for the acceptance criteria.

7.1. CRC Sensitivity Sample Size Justification

Assumption: $Sn_{CRC} \geq 85.0\%$, a sample size of 56 CRC achieves at least 90% power to reject the hypothesis of true CRC sensitivity of ≤ 65.0%.

If 56 CRC subjects are tested, then at least 44 need to be positive by the FMBT-CRC to reject H_0. The CI lower bound corresponding to 44/56 is 66.2%.

7.2. Non-ACN Specificity Sample Size Justification

Assumption: $Sp_{non\text{-}ACN} \geq 90.2\%$, a sample size of 454 achieves at least 90% power to reject the hypothesis of true non-ACN specificity of ≤ 85.0%.

If 454 non-ACN subjects are tested, then at least 401 need to be negative by the FMBT-CRC to reject H_0. The CI lower bound corresponding to 401/454 is 85.0%.

7.3. Non-ACN Negative Predictive Value Sample Size Justification

Assumption: $NPV_{non\text{-}ACN}$ – $Prevalence_{non\text{-}ACN} \geq 0.16\%$.

Given the critical values for $Sp_{non\text{-}ACN}$, Sn_{AA}, and Sn_{CRC}, in addition to the prevalence of non-ACN, AA, CRC, and estimated study size, a distribution of $NPV_{non\text{-}ACN}$ was generated, from which the 2.5th percentile was used for the acceptance criterion. Since sample size criteria will be met for those other endpoints, additional justification for sample size is not needed.

7.4. ACN Positive Predictive Value Sample Size Justification

Assumption: PPV_{ACN} – $Prevalence_{ACN} \geq 1.17\%$.

Given the critical values for $Sp_{non\text{-}ACN}$, Sn_{AA}, and Sn_{CRC}, in addition to the prevalence of non-ACN, AA, CRC, and estimated study size, a distribution of PPV_{ACN} was generated, from which the 2.5th percentile was used for the acceptance criterion. Since sample size criteria will be met for other endpoints, additional justification for sample size is not needed.

7.5. AA Sensitivity Sample Size Justification

Assumptions: $Sn_{AA} \geq 14.0\%$, a sample size of 1,929 AAs achieves at least 90% power to reject the hypothesis of true AA sensitivity of ≤ 11.5%.

If 1,929 AA subjects are tested, then at least 250 need to be positive by FMBT-CRC to reject H_0. The CI lower bound corresponding to 250/1,929 is 11.53%.

8. Statistical Methods

8.1. General Considerations Impacting the Data Analysis

- The Freenome testing results will be tabulated and data analysis will be performed on the compiled results as described in the study (and data unblinding) protocol.
- Categorical variables will be summarized by presenting the number and percent of observations in each category and the two-sided Wilson score 95% CI, unless otherwise

noted. When calculating percentages, the denominator will be the number of participants in the FAS, unless otherwise specified in the output.

- Continuous variables will be summarized using n (sample size), mean, standard deviation, median, percentiles (25, 50, and 75), minimum and maximum values, and 95% CI for the mean, unless otherwise noted.
- For the statistical analyses, the FMBT-CRC result will be coded as a binary categorical variable.
- Baseline information distributions will be tabulated separately for CRC, AA, ACN, non-ACN, NAA and NEG subjects.

8.2. P values and Confidence Intervals

For primary and secondary endpoints, the one-sided lower 97.5% confidence interval must exceed its respective acceptance criterion. For informational purposes, a p-value may be generated, corresponding to a one-sided test with a significance level of α = 0.025. In all other cases, unless otherwise specified, tests will use a nominal 0.05 level of significance, and 95% two-sided CIs will be presented for endpoints based on methods described in Section 6.2

Since all primary endpoints must be met for the primary objective to be achieved, no multiplicity correction is required.

Multiplicity is controlled for the secondary objective because the primary objectives are tested first, and the secondary objective is tested only if all primary objective success criteria are met.

8.3. Baseline Subject Characteristics

Subject demographics will be presented for the ALL, EN, FAS and SAS study populations. This will include the subject's age in years as a continuous variable, age stratified by ranges of clinical interest, biological sex, race and ethnicity, BMI, and lifestyle information (e.g. smoking history and alcohol use).

The age brackets (inclusive of both endpoints) of clinical interest that will be presented are [45 to 49], [50 to 54], [55 to 64], [65 to 74], [75 and greater], and separately, [45 to 54].

Age will be summarized using mean, standard deviation, median, interquartile range, minimum, and maximum values. For categorical variables (stratified age, race, ethnicity, etc.), the number and percentage of subjects in each category will be used.

8.4. Subgroup Analyses

Note: PREEMPT is not prospectively powered for subgroup analysis, nor is study alpha controlled for subgroup analysis. Reported analyses and p-values should be interpreted accordingly.

For subjects in the FAS, the condition prevalence (i.e. CRC, AA, Non-ACN) and clinical performance (unless otherwise noted, CRC sensitivity, AA sensitivity, and non-ACN specificity) of FMBT-CRC will be compared using Fisher-Freeman-Halton Exact tests for the following categorical variables (FDA 2017):

- Biological sex
- Race/ethnicity
- Categorical age brackets (note: for this analysis, a Cochrane-Armitage test for trend will be performed)
- Smoking status
- CS completion within 90 days vs. > 90 but ≤ 120 days after blood collection
- For CRC, cancer stage
- For CRC, location (proximal, distal and rectal)
- For AA, sub-type (2.1-2.5)
- For AA, location (proximal, distal and rectal)
- For AA, index lesion size (≤ 5, 6-9, 10-19, 20-29, and ≥ 30mm). (note: for this analysis, a Cochrane-Armitage test for trend will be performed)

The clinical performance of FMBT-CRC will also be analyzed via multivariate logistic regression (probability of a positive FMBT-CRC result as a function of listed variables) as follows:

- For NAA and AA (Categories 2-3), analysis of test positivity by index lesion size and location. The model will be repeated to also include age, biological sex, and race variables.
- For non-ACN, analysis of test positivity controlling for race, biological sex, and age.
- For CRC and AA (separately), analysis of test positivity, controlling for age, biological sex, race, index lesion size and lesion location.

Note: for multivariate logistic regression, Wald tests will be used to evaluate each factor for statistical significance, and age will be treated as a continuous factor.

8.5. Additional Analyses

1. Descriptive statistics for medical history, concomitant medication, adverse events, summary of blood test accessioning, processing, and findings, and summary of colonoscopy findings.
2. Diagnostic yield.
3. Report percentage of results with invalid tests.
4. Subject demographics and analysis of test performance (Sn, Sp, PPV, NPV, PLR and NLR) homogeneity by sites stratified by Science 37 and non-Science 37 sites, and US vs non-US sites.
5. Analysis of test performance (Sn, Sp, PPV, NPV, PLR and NLR) for the following age-based USPSTF (2021) Grade groups:
    - 5.1. USPSTF Grade “A” (50-75)
    - 5.2. USPSTF Grade “A+C” (50-85)
    - 5.3. USPSTF Grade “B+A” (45-75)
    - 5.4. USPSTF Grade “B+A+C” (45-85)
6. Comparison of age and sex distributions to US estimates.
7. NPV and PPV using U.S. IUP prevalence.

8. Direct rate standardization will be used to project the sensitivity (Sn_{ACN}, Sn_{CRC}, and Sn_{AA}) and specificity ($Sp_{non\text{-}ACN}$) of FMBT-CRC to the general population based on age brackets and biological sex. Standardization will be repeated, projecting for condition prevalence (based on age brackets and sex) to the general population, prior to projecting the sensitivity and specificity to the general population.

9. Number needed to screen per disease-positive finding (CRC, Stage I to III CRC, AA).

10. Benefit-harm analysis: sensitivity, specificity, PPV, NPV, and likelihood ratios have limited utility for guiding clinical decision-making and don't convey the impact of clinical application (Evans 2016). Additionally, actuarial-based frameworks such as BED-FRAME, that are designed to assess clinical impact, are not able to incorporate the dynamic relationship between natural history (i.e. the adenoma-carcinoma sequence) and guideline-based screening and colonoscopy surveillance. Because of these limitations, FMBT-CRC performance characteristics will be used in conjunction with a validated microsimulation model to assess lifetime comparative tradeoffs in harms (e.g. colonoscopy adverse events), burden (e.g. total colonoscopies), and benefit (e.g. life-years-gained), utilizing the same systematic approach to evaluate benefit-risk trade-offs as the United States Preventive Services Task Force (USPSTF) and Cancer Intervention and Surveillance Modeling Network (CISNET).

[redacted]

8.7. Demographics for Evaluated vs. Unevaluated Subjects

Subjects included in the EN set may be excluded from FAS for various reasons. Additionally, subjects enrolling prior to April 5, 2021 are not included in either the EN or the FAS. The distribution of categorical demographic variables (stratified age, race/ethnicity, etc.) will be descriptively compared to the corresponding distribution from the FAS set for the following:

- Enrolled subjects prior to April 5, 2021 (date used to define the EN and FAS set)
- Subjects included in EN with missing or incomplete CS results
- Subjects included in EN with missing FMBT-CRC results

Subject Accounting

There will be a detailed accounting of every subject in the EN cohort, noting their inclusion in FAS or the reason(s) for their exclusion from FAS.

8.8. Statistical Software

All tables, listings and figures will be primarily produced using SAS® Version 9.3 or later (SAS Institute, Cary, NC) and R version 4.2 or later (R Foundation for Statistical Computing, Vienna, Austria).

9. QA/QC and Data Handling

All data collection and monitoring methods are included within the Protocol Version 3, dated 09 April 2021.

Document Revision History

Table 7 - Revision History

Rev	Change	Author
1.0	Initial release	[redacted]

2.0	See Table 8	[redacted]
3.0	See Table 9	[redacted]
4.0	See Table 10	[redacted]
5.0	See Table 11	[redacted]

Table 8 - PL-00052 Rev 2 Summary of Changes

Rev 2 Section #	Change
Synopsis	Updated to add a brief statement that test results will be determined on two separate classification models.
Study Objectives	Updated to include PPV (for ACN) and NPV (for Non-ACN) as primary objectives per FDA feedback in [redacted] Included Fixed Sequence Tiered approach to Endpoint evaluation.
Section 1.1 Background	Updated to describe USPSTF recommendation of 1-3 year screening interval for screening modalities similar to FMBT-CRC. Updated to note that for purposes of statistical analysis, the definition of AA is changed to remove hyperplastic polyps ≥ 1.0 cm from AA category as per FDA feedback in [redacted].
Section 2 Device Description	Updated the device description for clarity regarding the FMBT-CRC Methylation assay.
Section 2.2 Subsystem Controls and Calibrators	Renamed section to "Subsystem Controls" because calibrators applied to the [redacted] subsystem, which is no longer applicable.
Section 2.4 FMBT-CRC-Multiomics Software Platform	Updated to describe classifier workflow with two separate models.
Section 3.1 Detection of ACN	[redacted]

Rev 2 Section #	Change
	[redacted]
Section 3.5 Classification	Updated to include classification model calculation for FMBT-CRC-Methylation model.
Section 4.4 Colonoscopy	Updated to include rationale for accepting patient samples that had CS within 90-120 days of blood collection.
Section 4.5 Study Duration	Removed in Rev 2 as this was repeat information from Section 4.4 Colonoscopy. All Section 4 sub-sections after 4.4 have been renumbered in SAP Rev 2.
Section 4.6 Central Pathology Review	Note that the central pathology review described has already occurred using classification previously provided. Freenome cannot change this section. Table 6 updated to differentiate hyperplastic polyps ≥1.0 cm from sessile serrated adenoma/polyp ≥1.0 cm by moving hyperplastic polyps ≥1.0 cm to a new NAA sub-category (3.3) for the purposes of the statistical analysis. These changes were intended to reflect FDA feedback to [redacted]
Section 4.7 Subject Disposition	Updated the subject disposition flowchart to better demonstrate subjects' disposition to different analysis sets and distinguish the exclusion reasons by blood sample issues versus colonoscopy/histopathology diagnosis issues.
Section 4.8 Protocol Deviations	Updated to clarify that deviation is from protocol defined procedure. Removed "visit not done" as a deviation as it is a duplicate to the "protocol-defined procedure".
Section 4.9 Blinding	Updated blinding definition to include blood test results that could bias the study.
Section 5.3 Safety Analysis Set	Updated the definition of Safety Analysis Set (SAS) to ensure subjects who had attempted blood collection are included for the adverse event analysis

Rev 2 Section #	Change
Section 6.1 Tiered Endpoints Families	New section added to describe Tiered Endpoint Families for FMBT-CRC Multiomics and Methylation Models as separate.
Section 6.2 Diagnostic Accuracy Estimation and Notation	Updated to replace Clopper-Pearson method with Wilson score method for analysis per FDA feedback in [redacted]
Section 6.3 Study Objectives	Updated to include PPV (for ACN) and NPV (for Non-ACN) as primary objectives per FDA feedback in [redacted] Updated to include Tiered Endpoint Family approach for the primary objectives.
Section 6.4 Study Hypotheses	Updated to describe two tiered analysis approach for FMBT-CRC Multiomics and Methylation models. Updated to move hypotheses for non-ACN NPV and ACN PPV from Secondary Objectives to Primary Objectives.
Section 6.5 Study Endpoints	Updated to replace Clopper-Pearson method with Wilson score method for analysis per FDA feedback in [redacted] Updated to include NPV for non-ACN and PPV for ACN as primary objectives per FDA feedback in [redacted]
Section 7 Sample Size Justification	Updated to replace Clopper-Pearson method with Wilson score method for analysis per FDA feedback in [redacted]
Section 7.1 CRC Sensitivity Sample size Justification	Updated to CI lower bounds corresponding to 38/48 as 65.74% instead of 65.01%. These values were updated based on feedback in [redacted]
Section 7.2 Non-ACN Specificity Sample Size Justification	Updated to CI lower bounds corresponding to 403/456 as 85.11% instead of 85.07%. These values were updated based on feedback in [redacted]
Section 7.5 AA Sensitivity Sample	Updated to CI lower bounds corresponding to 21/131 is 10.73% instead of 10.21% These values were updated based on feedback

Rev 2 Section #	Change
size Justification	in [redacted]
Section 8.1 General Considerations Impacting the Data Analysis	Updated to replace Clopper-Pearson method with Wilson score method for analysis per FDA feedback in [redacted] Removed NEG 4.4 Category.
Section 8.2 P Values and Confidence Interval	Updated to replace Clopper-Pearson method with Wilson score method for analysis per FDA feedback in [redacted] Updated to describe the two tiered endpoint family analysis approach.
Section 8.4 Subgroup Analysis	Updated to clarify the method used for statistical significance, specified that age will be treated as a continuous factor, and removed a redundant model

Table 9 - PL-00052 Rev 3 Summary of Changes

Rev 3 Section #	Change
Title	In title, replaced “Multiomics” with “Methylation”
List of Abbreviations and Definitions	Updated FMBT-CRC to FMBT-CRC/FMBT-CRC Methylation for clarity.
List of Abbreviations and Definitions	Added a definition for Enrolled* subjects. Added a definition for Index Lesion.
List of Abbreviations and Definitions	Updated the definition of FRNM-SP for clarity.
Synopsis Study Design	Deleted reference to “two separate classification models” and references to a “multiomics” test. Deleted reference to “tiered endpoint family”. Deleted the section “Fixed Sequence (Serial Gatekeeping) Tiered Approach to Endpoint Evaluation.” Deleted Figure 1, which described Fixed Sequence (Serial Gatekeeping) approach.

Rev 3 Section #	Change
Section 1.1 Background	Added text to describe the reason for re-classifying Category 2.4 hyperplastic polyps was due to FDA feedback.
Section 2 Device Description	Deleted reference to "multiomics", "[redacted]", and "[redacted]" subsystem.
Section 2 Device Description	Added FMBT Blood Collection Kit and FMBT Pre-Analytical Subsystem to the list for the overall test system.
Figure 1	Updated figure to remove [redacted] assay.
Figure 2	Updated to remove "[redacted] assay."
Section 2.2 [redacted] subsystem	Deleted section, as it refers to the [redacted] subsystem.
Table 2	Deleted, since it applies to the [redacted] subsystem.
Table 3	Deleted, since it applies to the [redacted] subsystem.
Table 5	Deleted, since it applies to the [redacted] subsystem.
Section 2.4 FMBT-CRC-Multiomics	Deleted, since multiomics will not be tested with this Plan.
Figure 3	Deleted, since it applies to multiomics, and multiomics will not be tested with this Plan.
Figure 4	Deleted, since it applies to multiomics, and multiomics will not be tested with this Plan.
Section 2.5 FMBT-CRC—Methylation Software Platform	Changed the section title to "FRNM Software Platform (SP" to align with correct nomenclature. Updated this section to align with actual device description and to make language more clear for FMBT-CRC-Methylation, given deletions in section 2.4.
Section 3.1 Detection of ACN	Deleted reference to FMBT-CRC Multiomics Classification Model. Updated verbiage pertaining to FMBT-CRC Methylation Classification Model.
Section 3.2 CpG	Updated entire section describing how CpG Methylation Score is

Rev 3 Section #	Change
Methylation Score	derived by providing more specifics of the process.
Section 3.3 [redacted]	Section deleted, since it applies to [redacted] score.
Section 3.3 Model ensemble	Added a Model ensemble section to describe the training, tuning, and testing procedure for FMBT-CRC-Methylation model.
Section 3.4 Integrating Methylation and [redacted] Score into FMBT-CRC-Multiomics System Level Score	Section deleted, as it applies to integrating Methylation and [redacted] Subsystems.
Section 3.5 Classification (now Section 3.4)	Section updated to only apply to Methylation classification. Provided updated language and symbols for comparing Score to Cutoff.
Section 4 Scope of the Analysis	Improved the grammar and readability of the section. Removed redundant verbiage.
Section 4 Scope of the Analysis	Clarified enrollment cutoff date to read "on or after April 5th" or "April 5th 2021 (inclusive) cutoff" so language aligns with existing section 7.12 language that defines exclusions as prior to April 5th.
Section 4.4 Colonoscopy	Updated language to make it clear the adjectives "poor" and "inadequate" applied to bowel preparation.
Section 4.6 Central Pathology Review	Added description of index lesion.
Section 4.7 Subject Disposition Figure 7 and bullet points below	Updated definition of "All Enrolled" subjects to include any subject enrolled in PREEMPT. Introduced "Enrolled*" term to delineate from subjects enrolled prior to April 5th, 2021. Changed "blood sample not obtained" to "blood sample not available" Changed order of bullet points of "Death" and "Adverse Event" Changed "Unevaluable colonoscopy result" to "Unevaluable colonoscopy result for non-ACN subjects". Removed "Occult IBD found on colonoscopy" from "Subjects

Rev 3 Section #	Change
	excluded due to colonoscopy or histopathology results" as these subjects could be considered part of IUP. Updated footer to "on or after April 5th, 2021" to define *Enrolled subjects and added a footer about the SAS group.
Section 4.7 Subject Disposition	Clarified protocol deviation and blood sample failure language.
Section 4.8 Protocol Deviations	Updated protocol deviations section to align with Tables, Listings, and Figures
Section 4.9 Blinding	Added document number for Sponsor's blinding plan.
Section 5 Analysis Populations	Added Enrolled* population and clarified enrollment cutoff date to read "on or after April 5th" so language aligns with existing section 7.12 language that defines exclusions as prior to April 5th.
Section 6.1 Tiered Endpoint Families	Deleted this section, since two models will not be evaluated with this Plan.
Section 6.2 Diagnostic Accuracy Estimation and Notation	Corrected the calculations for PLR_CRC, PLR_AA, NLR_CRC, and NLR_AA so when they are used with corresponding prevalences, the resulting PPV and NPVs will align with outcomes of the formulas.
Section 6.2 Diagnostic Accuracy Estimation and Notation	Aligned the PLR, NLR, PPV, and NPV confidence interval approach to the Score method, per FDA (2017) guidance.
Section 6.3 Study Objectives	Replace "positive predictive value" with "PPV" to make the bullet point more consistent with its NPV counterpart.
Section 6.4 Study Hypotheses	Deleted reference to evaluating two tiers of endpoint families, since two models will not be evaluated.
Section 6.4 Study Hypotheses	Removed hypothesis-testing language and replaced with acceptance criteria language. Updated powering and sample size estimates based on performance assumptions and simulation-based approach for predictive values. Predictive value (NPV and PPV) endpoints now based on delta compared to observed

Rev 3 Section #	Change
	prevalence (for non-ACN and ACN, respectively).
Section 7 Sample Size Justification	Updated Sample Size Justification section to align with Section 6.
Section 7.1 CRC Sensitivity Sample Size Justification	Updated sample size requirements with new assumptions. Added Score confidence interval language.
Section 8.1 General Considerations Impacting the Data Analysis	Updated the quantile values that will be summarized for continuous variables.
Section 8.2 P values and Confidence Intervals	Deleted reference to two tiers of endpoint families.
Section 8.3 Baseline Subject Characteristics	Added "EN" analysis set. Removed "family history" from baseline subject characteristics, since family history of CRC is a study exclusion criteria. Added BMI and lifestyle information to baseline subject characteristics.
Section 8.4 Subgroup Analyses	Added a disclaimer about interpretability of subgroup analyses and added specifics about the statistical tests that will be performed. Added reference to FDA's guidance for subgroup evaluation for Medical Devices. Clarified that Fisher-Freeman-Halton Exact test and Cochrane-Armitage test for trend will be used for subgroup evaluation.
Section 8.5 Ad-hoc analyses	Changed title of section to "Exploratory analyses"
Section 8.5 Ad-hoc analyses	Added verbiage in the direct standardization section to align with commitments made to FDA.
Section 8.5 Ad-hoc analyses	Added an ad-hoc analysis for quantifying lifetime burdens and benefits, using methods that are similar to USPSTF/CISNET. Added Evens 2017 reference.

Rev 3 Section #	Change
Section 8.5 Ad-hoc analyses	Updated the order of ad-hoc analyses for readability.
Section 8.5 Ad-hoc analyses	Updated verbiage of “maximum polyp size” to “index lesion size” for clarity.
[redacted]	[redacted]
Section 8.7 Demographics vs Unevaluated Subjects	Changed “ALL” to “EN” to reflect the correct analysis set for these analyses.

Table 10 - PL-00052 Rev 4 Summary of Changes

Rev 4 Section #	Change
Section 6.3 Study Acceptance Criteria	Updated specificity acceptance criterion to 85.0% (from 89.0%) Updated non-ACN NPV acceptance criterion to 0.16% (from 0.26%) Updated ACN PPV acceptance criterion to 1.17% (from 2.19%)
Section 6.4 Study Endpoints	Updated specificity acceptance criterion to 85.0% (from 89.0%) Updated non-ACN NPV acceptance criterion to 0.16% (from 0.26%) Updated ACN PPV acceptance criterion to 1.17% (from 2.19%)
Section 7.2 Non-ACN Specificity Sample Size Justification	Updated specificity acceptance criterion to 85.0%. Updated corresponding sample size to 454. Updated associated critical value to 401/454.
Section 7.3 Non-ACN Negative Predictive Value Sample Size Justification	Updated non-ACN NPV acceptance criterion to 0.16% (from 0.26%)
Section 7.4 ACN Positive Predictive Value Sample Size Justification	Updated ACN PPV acceptance criterion to 1.17% (from 2.19%)
Section 8.5 Exploratory Analysis	Section was renamed to Additional Analyses
Section 8.5 Exploratory Analysis	Added exploratory analysis for test performance by age-based USPSTF (2021) Grade groups.

Table 11 - PL-00052 Rev 5 Summary of Changes

Rev 5 Section #	Change
List of Abbreviations and Definitions	Updated definition of meCpG from "Cpg Methylation" to "A methylated cytosine followed by a guanine"
List of Abbreviations and Definitions	Corrected the definition of NEG to align with the other sections of the protocol
Section 8.4 Subgroup Analyses	Corrected AA subgroups from "2.1-2.4" to "2.1-2.5" to align with the other sections of the protocol

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