Non-Clinical Verification and Clinical Validation of BeScreened™-CRC, a Blood-Based In Vitro Diagnostic Multivariate Index Assay for the Detection of Colorectal Cancer in Screening Non-Compliant Patients

Abstract

Despite decades of promoting awareness, education and increasing availability of colorectal cancer (CRC) screening, approximately 35 million Americans, or 35% of the eligible population, remain unscreened for CRC . It's estimated that 65% of all CRC deaths (32,670 of 50,260) and 75% of the treatment costs ($12 of $16 billion / year) stem from this unscreened population. Beacon Biomedical has developed BeScreened™-CRC, an easy, accurate and affordable blood-based test for earlier CRC detection designed to help navigate these "hard-to-reach" unscreened patients into a screening program, enable earlier CRC detection and, in doing so, help reduce CRC mortality due to earlier cancer detection. Founded on three tumor-associated protein markers (Cripto-1, CEA and a marker of cancer associated with extracellular matrix modifications), BeScreened-CRC is a clinically validated CLIA Laboratory Developed Test (LDT). In a case-control study of 110 subjects [45 colorectal cancer cases (Stage I – Stage IV) and 65 controls] BeScreened™-CRC demonstrated a sensitivity of 91%, specificity of 86% and an overall accuracy of 94.5% (area under the ROC curve) making BeScreened-CRC a highly accurate blood test for colorectal cancer screening.

Background

Despite being one of the most preventable forms of cancer, colorectal cancer (CRC) remains the second deadliest cancer claiming more than 50,260 lives in 2017.[1] Approximately 65% (more than 30,000) of those deaths stem from the 35% of screening eligible patients who are non-compliant with existing CRC screening tests,[2] such as colonoscopies and fecal-based testing. 75% of these patients have both insurance coverage and a primary healthcare contact. [3,4] Yet, they avoid screening for a variety of reasons, including that they find the current screening procedures painful, disgusting or simply inconvenient to be worthwhile, and decades of increased awareness, education and expanded test availably have failed to overcome these objections.

However, these patients can be reached. Both common sense and patient preference studies suggest that most of these reluctant individuals are perfectly willing to be screened with a patient friendly option. In a 2014 study, Adler, et al, offered screening colonoscopies to 172 patients, 109 of which refused (63%). When the latter were offered alternative screening methods; 3 subjects (3%) refused all options remaining unscreened, 16 opted to take a fecal-based screen (15%), and 90 of the remaining 93 unscreened subjects were willing to take a blood-based screen (83%).[5]

Having demonstrated an increase in compliance to 97%, Adler's study suggests an accurate blood-based screening test is the key to achieving high levels of colorectal cancer screening compliance.

Study Objectives

Beacon Biomedical (a CLIA accredited laboratory) sought to clinically validate a blood-based assay (BeScreened™-CRC) for colorectal cancer screening. The assay is intended patients who are unable or unwilling to participate in current CRC fecal-based testing or colonoscopy CRC screening procedures.

About BeScreened™-CRC: BeScreened-CRC is an ELISA-based multi-analyte assay with an algorithmic analysis (MAAA) built upon three, well established blood-based CRC associated protein biomarkers; an oncoprotein called teratocarcinoma derived growth factor-1 (TDGF-1, Cripto-1);[6,7] carcinoembryonic antigen (CEA);[8,9] and an extracellular matrix (ECM)[10,11] protein involved in early stage tumor stroma changes. The quantitative assay results from each of the three biomarkers are processed through Beacon's proprietary relational algorithm to produce a BeScreened-CRC Index. Patient BeScreened-CRC Indexes that fall within the normal range are reported as negative for the potential presence of CRC with a recommendation to stay compliant with their healthcare well-checks and screening. Patients with a calculated BeScreened-CRC Indexes outside the normal range are reported as positive for the potential presence of CRC with a recommendation to follow-up with their physician to schedule a screening colonoscopy.

Regulatory Background: Newly developed laboratory developed tests (LDTs), such as BeScreened™-CRC, are regulated under the CMS' Clinical Laboratory Improvement Amendments, CFR

493.1253(b)(2) that requires the establishment of (i) accuracy, (ii) precision, (iii) analytical sensitivity, (iv) analytical specificity (including interfering substances), (v) reportable range of test results for the test system, (vi) reference intervals (normal values) and (vii) any other characteristic required for predictable test performance.

Summary of Non-Clinical Verification

Each BeScreened-CRC marker was non-clinically verified according to a CLIA compliant testing plan (see Table below) pre-approved by Clinical Pathology Associates, PLC; the largest physician-owned pathology practice Arizona, providing laboratory services and CAP and CLIA lab oversight.

Test	Criteria	Cripto-1 Results	CEA Results	ECM Results
Accuracy	80%-120%	95%	90%	90%
Precision	<15% CV	3.80%	4.38%	3.40%
Repeatability	<15% CV	6.77%	9.87%	3.53%
Calibration	<15% CV	-0.40%	-0.30%	5.30%
Time Temp	<15% CV	7.74%	6.69%	4.33%
Interference	<15% CV	4.03%	10.60%	3.36%

Analytical Accuracy results for each of the three constituent biomarkers were within the predefined acceptance criteria with an average accuracy of 95%, 90% and 90% for Cripto-1, CEA and ECM, respectively. Each was determined by spiking known amounts of recombinant protein into sample matrix at three different concentrations. All measured accuracies were within the required 80% - 120% recoveries.

Precision was determined to be 3.8%, 4.38% and 3.40% when eight replicates of three concentrations were tested for Cripto-1, CEA and ECM, respectively. Precision for all conditions were within the required 15% CV.

Repeatability was assessed at 6.77%, 9.87%, and 3.53% for Cripto-1, CEA and ECM, respectively, inclusive of inter-operator, inter-day and inter-plate results.

Pre-Analytical Evaluations: The *Time/Temperature* stability of clinical samples was determined using a freshly drawn pool of serum from healthy controls. The pooled serum was sub-aliquoted into a control samples, (immediately frozen at -80C^{o}) and individual test samples. Test samples were held at (i) 4C^{o}, (ii) room-temperature and (iii) 35C^{o} for times ranging from 2 to 96 hours prior to freezing for each subset. The average value from at least 5 replicates of each test condition were compared to the control value.

In a similar fashion, the effects of *Potentially Interfering Substances* were quantified by sub-aliquoting freshly-drawn serum samples into a control sample and a set of test samples. The control sample was unadulterated, while pathological levels of bilirubin (free and bound), emulsified fat (lipemic sample), lysed red blood cells (hemolyzed samples) were added to a set of test samples. Sodium citrate, EDTA and heparin were added to a second set of test samples to mimic improper use of plasma tubes for sample collection or transport. Samples were held at -80C^{o} for a minimum of 48 hour prior to analysis.

Non-Clinical Verification Outcome: As a result of the pre-analytical testing, samples held for more than 48 hours at room temperature or greater than 96-hours at 4C are rejected. Also, samples with any evidence of exposure to sodium citrate or EDTA, along with those that are visibly hemolyzed or lipemic upon receipt will be rejected.

All three of BeScreened-CRC constituent biomarkers were fully verified for CLIA non-clinical performance and have been certified and approved for clinical use.

Summary of Clinical Validation

The accuracy of BeScreened-CRC was determined in a case-control study of 110 subjects, inclusive of 45 colorectal cancer cases (Stage I – IV) and 65 controls. The study was conducted in three phases; a training phase, a validation phase and a final retrospective analysis.

Subjects: Serum samples from CRC cases and controls were obtained from the following commercial bio-repositories; Asterand Bioscience (Detroit, MI), Conversant Biologics (Huntsville, AL) and BioServe Biotechnologies (Beltsville, MD). Biorepository samples included all 45 cases and 34 control subjects. An additional 31 control samples were obtained prospectively from community volunteers. All bio-repository samples were collected with IRB approval and written consent was obtained from all community volunteers. A total of 45 subjects were men and 65 were women. Both cases and controls were limited to subjects of screening age (45-80 years old). Cancer cases were

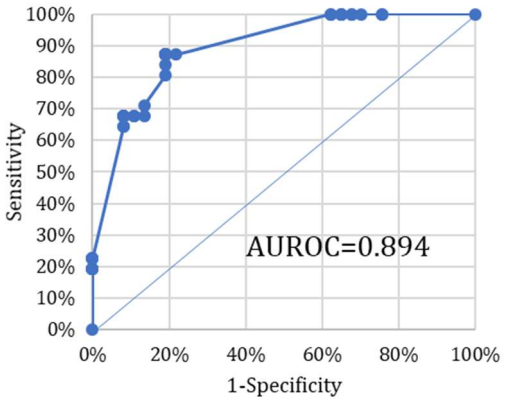
older than controls with an average age of 64.6 (stdev 9.25) for cases and 57.9 (stdev 7.97) for controls ($p<0.05$). The majority (91%) of cases and controls were Caucasian, with 6.3% and 2.7% of subjects being African American or Hispanic.

Cohort	n	Average Age	Stdev
Controls	65	57.9	7.97
Cases	45	64.6	9.25
Stage I	9	69.6	3.85
Stage II	14	63.8	9.01
Stage III	15	67.8	8.62
Stage IV	3	55.3	3.06
Unknown	4	54.3	9.22
Total	110	60.4	8.96

BeScreened-CRC Data Modeling: Data modeling took place in three phases; Initial Training & Model Development, Validation with Independent Sample Set, and Full Retrospective Model Development.

Initial Training & Model Development: The initial training data set included quantitative values for Cripto-1, CEA and ECM on a set of 28 cases and 37 controls. All markers produced statistically significant results individually ($p<0.05$). Combined into a predictive algorithm, the training set was able to statistically differentiate cases from controls ($p<7x10^{-11}$) with 87% sensitivity, 81% specificity and an AUROC of 0.89.

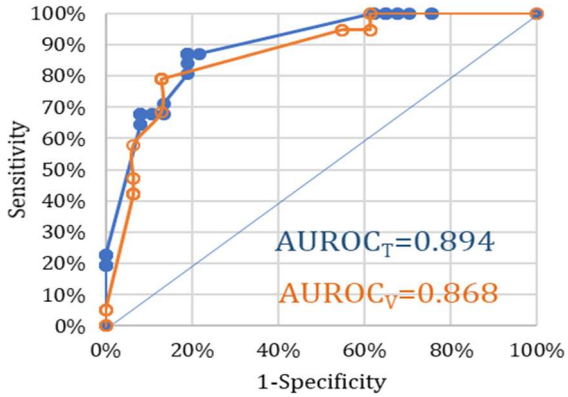
Training Model Accuracy



Independent Validation: Following the development of the Training Model; a second independent set of samples consisting of 17 cases and 28 controls were analyzed for the BeScreened-CRC oncoproteins and indexed using the Training Model.

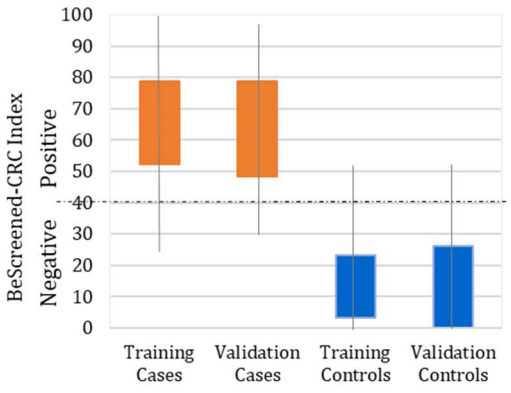
Concurrence of Training and Validation: As expected, sensitivity (87% vs. 79%), specificity (81% vs. 87%) and AUROC (0.894 vs. 0.868) changed somewhat from the training data to the independent validation data. These changes were, however, small given (i) the size of sample sets, (ii) the use of an independent validation set of biospecimens, (iii) the additional inclusion of biospecimens in the validation set from a new vendor not previously used, and (iv) the inclusion of prospectively collected samples that are fully representative of the intended clinical use population. As noted in the chart below, the overall accuracy, as measured by the AUROC, changed by just 0. 026, or 2.6%.

Training & Validation Set Accuracy

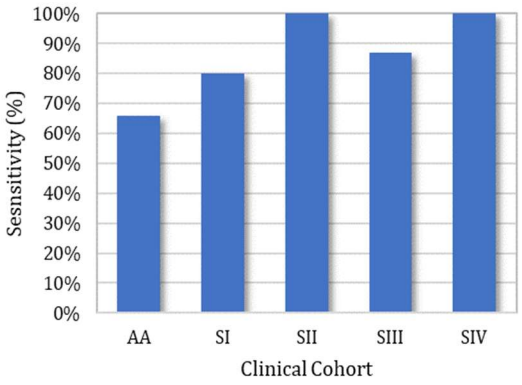


The consistency between the training model and the independent validation is also evident in the box and whiskers plot shown below.

BeScreened-CRC Model Validation



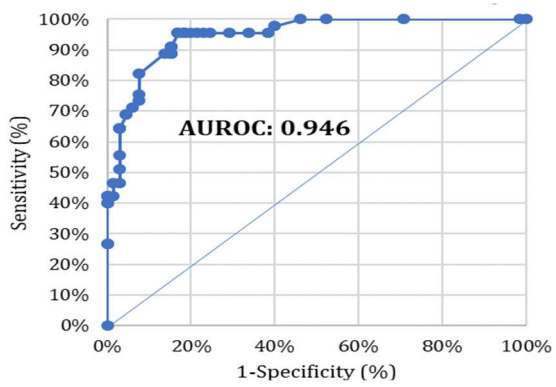
BeScreened-CRC Stage Sensitivity



Final Retrospective Data Model

A final retrospective model was generated using the full data; i.e. all 45 cancers and 65 controls. As in the training model, all variables were statistically significant ($p<0.05$) employing univariate analysis separating cases from controls. As expected, the full retrospective model improved upon the training and validation sets, producing a final sensitivity for BeScreened-CRC of 91%, specificity of 86% and an overall accuracy (AUROC) of 0.946. Compared to the training set, sensitivity improved from 87% to 91%, specificity from 81% to 86% and overall accuracy (AUROC) from 0.894 to 0.946.

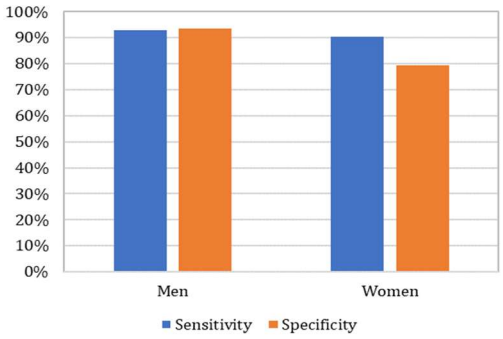
BeScreened-CRC Final Accuracy



Sensitivity by Stage: The BeScreened-CRC results were stratified by cancer stage. As seen in the chart below, BeScreened-CRC maintains a high sensitivity across all stages of cancer ranging from 80% in Stage I, 87% in Stage III and 100% in Stages II and IV, with differences between the stages not reaching statistical significance (Stage I vs. Stage II $p=0.37$). Advanced adenoma (n=6), while not included in the modeling, were indexed using the model demonstrating >60% sensitivity to their detection.

Accuracy by Gender: BeScreened-CRC was accurate in both men in women (see chart below). When stratified, BeScreened-CRC was 93% sensitive and 94% specific in men vs. 90% sensitive and 79.4% specific in women.

BeScreened-CRC Accuracy by Gender



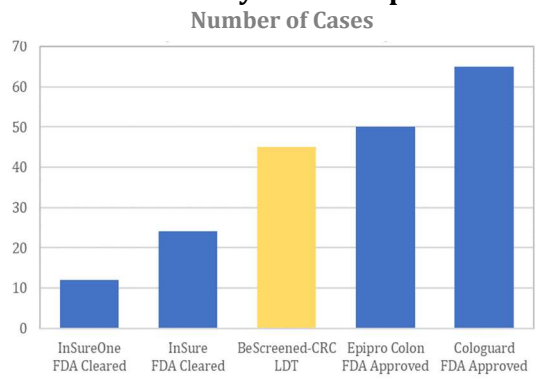
Study Strengths and Limitations

BeScreened-CRC's clinical validation study addressed the typical limitations associated with such a case-control design; i.e. the potential for bias and incomplete information. Bias potential typically exists in the selection of both controls and cases possibly skewing the latter toward the easiest to identify (e.g. later stage vs. early stage). These biases were mitigated in three ways. First, each biorepository's control samples needed to be qualified as statistical equivalent to prospectively collected controls (our gold standard) as an assurance of appropriate sample collection and handling. Once qualified, all sourced samples (cases and controls) from that biorepository were used.

Second, study cases were well distributed with 53% early stage cases (Stage I -II) and 47% later stage cases (Stage III - IV). Additionally, the lack of control subjects' screening history also introduces potential negative bias. Based on a 60% screening rate and an 8% incident rate of advanced adenomas in an unscreened population, it is estimated that 1-3 control subjects had undetected advanced adenomas (1.5% - 4.5%); which intrinsically creates a potential under-estimation of the test's specificity.

Finally, the current 110 case-control study far exceeds the CLIA validation requirements for an LDT approval. While a small study size (n) in total, the study included as many CRC cases (n) comparably as clinically used in many of the FDA CRC screening validation studies noted in the chart below.

Clinical Study Case Comparison

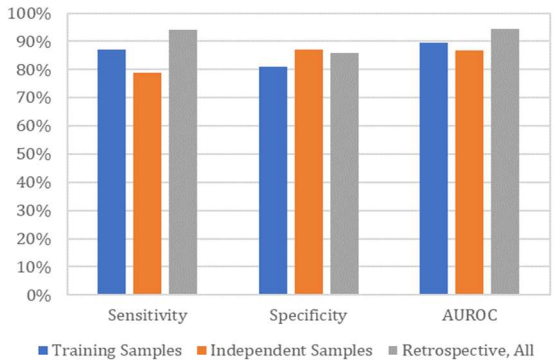


Continuing Research: To gain further insight on the current test's performance and enhance the indexing algorithm, additional studies and algorithmic refinements will be conducted on BeScreened-CRC. This would include, but be limited to, enhanced data analytics evaluations, controlled trials conducted by Beacon Biomedical and the collection and evaluation of post-market surveillance data conducted in conjunction with larger medical practices. Controlled trials will provide the most accurate data for hundreds of subjects, and the post market surveillance will provide real-world-evidence of BeScreened-CRC's larger clinical utility.

Summary of BeScreened-CRC Findings

BeScreened-CRC exceeded all the non-clinical validation requirements under CLIA and was also clinically validated over three phases of modeling with consistently high levels of performance at each step. Results were consistently high when stratified by cancer stage and subject gender, and achieved a final, full retrospective 91% sensitivity, 86% specificity and AUROC accuracy of 94.5%.

BeScreened-CRC Validation Summary



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