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Analytical validation of a novel multiplex test for detection of advanced adenoma and colorectal cancer in symptomatic patients

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ABSTRACT

Early detection of colorectal cancer (CRC) is key to reducing associated mortality. Despite the importance of early detection, approximately 40% of individuals in the United States between the ages of 50–75 have never been screened for CRC. The low compliance with colonoscopy and fecal-based screening may be addressed with a non-invasive alternative such as a blood-based test. We describe here the analytical validation of a multiplexed blood-based assay that measures the plasma concentrations of 15 proteins to assess advanced adenoma (AA) and CRC risk in symptomatic patients. The test was developed on an electrochemiluminescent immunoassay platform employing four multi-marker panels, to be implemented in the clinic as a laboratory developed test (LDT). Under the Clinical Laboratory Improvement Amendments (CLIA) and College of American Pathologists (CAP) regulations, a United States-based clinical laboratory utilizing an LDT must establish performance characteristics relating to analytical validity prior to releasing patient test results. This report describes a series of studies demonstrating the precision, accuracy, analytical sensitivity, and analytical specificity for each of the 15 assays, as required by CLIA/CAP. In addition, the report describes studies characterizing each of the assays' dynamic range, parallelism, tolerance to common interfering substances, spike recovery, and stability to sample freeze-thaw cycles. Upon completion of the analytical characterization, a clinical accuracy study was performed to evaluate concordance of AA and CRC classifier model calls using the analytical method intended for use in the clinic. Of 434 symptomatic patient samples tested, the percent agreement with original CRC and AA calls was 87% and 92% respectively. All studies followed CLSI guidelines and met the regulatory requirements for implementation of a new LDT. The results provide the analytical evidence to support the implementation of the novel multi-marker test as a clinical test for evaluating CRC and AA risk in symptomatic individuals.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed malignancy in both men and women in the United States, with an estimated 95,520 new cases predicted in 2017 [1]. The CRC survival rate would increase if more patients were identified at early stages of the disease [2]. The U.S. Preventive Services Task Force (USPSTF) recommends screening for CRC in average-risk, asymptomatic adults aged 50–75 years [3]; however, according to the National Health Interview Survey [4] only ~60% of individuals between the ages of 50–75 years are tested. The low compliance rate for colonoscopies and fecal-based tests suggests the need for an alternate non-invasive blood-based test.

We recently described the discovery of a new blood-based CRC test for symptomatic patients [5]. This test provides a means to reach an intent-to-test (ITT) population of symptomatic patients who decline the standard screening options, thus providing an alternate approach to enhance colonoscopy compliance and improve detection of early stage CRC. The CRC test had sensitivity/specificity of 0.80/0.83, positive predictive value (PPV) of 36.5%, and negative predictive value (NPV) of 97.1%. An advanced adenoma (AA) test developed using the same dataset had sensitivity/specificity of 0.44/0.80, PPV of 15%, and NPV of 94.7% (unpublished results). In the current paper, we describe the analytical validation of a 4-panel set of multiplexed electrochemiluminescent immunoassays used to develop and implement a

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blood-based test that combines our CRC and AA tests in a laboratory developed test (LDT).

Early studies from our laboratory described the discovery of a CRC test based on multiple singleplex chemiluminescent ELISA assays [6]. We later moved to multiplexed electrochemiluminescent immunoassays, with which new studies were performed to determine the optimal analytes and disease classifier parameters in the ITT population [5]. The electrochemiluminescent immunoassays were selected for the second generation tests because they provided a more flexible approach to creating custom multiplex immunoassays with the advantages of specimen conservation, high throughput analysis, and time and cost efficiency. Furthermore, the electrochemiluminescent assays provided excellent analytical sensitivity at pg/mL level detection limits, enabling 3–4 log dynamic ranges.

The 15-protein multi-marker CRC and AA test identified from our biomarker discovery and validation studies (Table 1) were intended for clinical use. As an in vitro diagnostic test manufactured in-house to assess CRC and AA risk, it is categorized by the Food and Drug Administration (FDA) as a high-risk LDT, and prior to clinical testing it must meet Clinical Laboratory Improvement Amendments (CLIA) requirements (42 CFR Part 493) [7] as well as College of Americal Pathologists (CAP) analytical requirements. For a high-risk LDT, CLIA and CAP require that the clinical laboratory demonstrate test performance specifications including precision, accuracy, analytical sensitivity and analytical specificity. This paper presents the findings of those studies as well as freeze-thaw testing, parallelism testing, spike recovery, dynamic range characterization, and the effect of interfering substances for each of the 15 biomarkers in the CRC and AA test. Having successfully met the CLIA/CAP regulatory requirements, we provide the required evidence to support the clinical implementation of this test as a LDT for the intended use of evaluating CRC and AA risk in symptomatic patients.

2. Experimental

2.1. Technology overview

We used sandwich electrochemiluminescent immunoassay technology from Meso Scale Discovery (MSD, Gaithersburg, MD, USA), specifically choosing the MSD U-PLEX platform [8]. In the U-PLEX assay, biotinylated capture antibodies are coupled to spot-specific linkers. These U-PLEX linkers with attached capture antibodies self-assemble onto the U-PLEX plate at the spot position specific to the linkers. After analytes in the sample bind to the capture reagents, detection antibodies conjugated with electrochemiluminescent ruthenium reporter molecules bind to the analytes to complete the sandwich immunoassay. Upon electrical stimulation via the cathode coated plate, the detection antibodies emit light that is directly proportional to the concentration of captured analyte.

2.2. Materials

2.2.1. Assay reagents

The capture and detection antibodies used in each of the 15 immunoassays (Supplementary Table 1) were identified following extensive assay feasibility studies (outside the scope of this manuscript). These studies included dilution linearity and spike recovery testing as well as antibody cross reactivity testing to confirm the compatibility of antibodies within a panel. When available, human derived purified native protein was selected as the standard, otherwise recombinant proteins were selected as standards (Supplementary Table 1). The 15 CRC and AA protein biomarkers were measured on four multiplexed panels (A–D), grouped together based on the plasma dilution requirements for each analyte (Table 1). The U-PLEX plates (with 2-, 3-, 4- or 6-activated spots), linkers, and Stop Solution were all purchased from MSD. PBS-T (Phosphate Buffered Saline with Tween-20, Sigma, St.Louis, MO) was used as assay wash solution for all panels. Sample and assay diluents containing proprietary additives as well as the Read Buffer T, required to catalyze the electrochemiluminescence reaction, were also purchased from MSD (Supplementary Table 2).

2.2.2. Assay quality control samples

Plasma-based quality control samples were built before the start of analytical validation. For each assay, a low-concentration quality control (LQC) and a high-concentration quality control (HQC), targeting the lower and upper clinical reportable range (CRR) (see below) of each assay, were used in each experiment to determine the analytical run quality. More than 500 individual plasma samples (Bioreclamation IVT, Westbury, NY), using 125 uL per sample, were screened in each of the assays to identify suitable control samples. LQCs were generated by pooling multiple plasma samples; HQCs required pooling of plasma followed by spiking with protein standards to generate sufficient volumes of control material with analyte concentration at the upper end of the assay range. Multiplexed HQC and LQC samples were built for each panel, then aliquoted and stored at −80 °C as single-use vials.

In addition to HQC and LQC samples, a single Process Quality Control (PQC) sample was added to each plate to monitor all aspects of the assay workflow. The PQC sample had an endogenous concentration within the CRR of each assay and was generated from pooled plasma samples (Bioreclamation IVT, Westbury, NY), aliquoted into single-use vials and stored at −80 °C. The acceptable concentration limits for the PQC were established for each assay from repeated measurements over a minimum of 10 days with at least three technical measurements per day (data not shown).

2.2.3. Plasma samples

The 500 individual plasma samples (Bioreclamation IVT, Westbury, NY) previously screened for building quality controls were subsequently assigned for use in the analytical validation experiments. For the analytical studies requiring samples spanning the CRR (see Section 2.3.3.2), power analysis was performed prior to the start of validation, to calculate the minimum sample size required in each of the four multiplexed panels (A–D) to obtain statistically valid results. Based on power analysis, a minimum of 74 plasma samples were selected to run against panels A, B and D and 111 plasma samples were selected for panel C. Panel C required a larger number of samples because one panel C assay – CO9 – had slightly higher inter-day variability than others. Simulations showed that the observed variability would have insignificant impact on classifier clinical calls. For a subset of assays, spiking the plasma with protein standards was required to span the higher concentrations of the CRR.

The clinical accuracy study used plasma samples that had been previously characterized in earlier classifier validation studies [5]. Powering of this study is described below (Section 2.3.3.8).

2.3. Methods

2.3.1. Multi-analyte assay workflow

Batches of 2-, 3-, 4- and 6-spot U-PLEX plates (panels A–D) and spot-specific U-PLEX linkers were manufactured and distributed by MSD under ISO 9001:2008 and ISO 13485:2003 certified quality management systems. Each plate type was prepared in-house by coating 15–20 h (overnight) ahead of sample plating, using the plate-specific biotinylated capture antibodies coupled to the U-PLEX linkers. Each 96-well assay plate accommodated duplicate