Cologuard Plus™

Instructions for Use

mt-sDNA-based Colorectal Cancer Screening Test

IVD

Rx Only

LBL-11104_IFU
Rev. 3

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1 Intended Use and Indications for Use

The Cologuard Plus™ test is a qualitative in vitro diagnostic test intended for the detection of colorectal neoplasia-associated DNA markers and for the presence of occult hemoglobin in human stool. The Cologuard Plus test is performed on samples collected using the Cologuard Plus Collection Kit. A positive result may indicate the presence of colorectal cancer (CRC) or advanced precancerous lesions (APL) and should be followed by colonoscopy. The Cologuard Plus test is indicated to screen adults 45 years or older, who are at average risk for CRC. The Cologuard Plus test is not a replacement for diagnostic colonoscopy or surveillance colonoscopy in high-risk individuals.

The Cologuard Plus test is performed at Exact Sciences, Madison, WI.

2 Summary and Explanation of the Test

The Cologuard Plus test is an in vitro diagnostic device designed to analyze a patient's stool for the presence of DNA and hemoglobin markers which may indicate the presence of CRC or APL. Specifically, two independent categories of biomarkers are targeted and provide an additive association for the detection of CRC and pre-malignant neoplasms. The combined result/composite score gives a qualitative result, Positive (abnormal) or Negative (normal) which is associated with increased or decreased likelihood of CRC and APL.

The first category of biomarkers detects epigenetic DNA changes characterized by aberrant gene promoter region methylation. The specific methylated gene targets include ceramide synthase 4 gene (*LASS4*), leucine-rich repeat-containing protein 4 gene (*LRRC4*), and protein phosphatase 2 regulatory subunit B' gene (*PPP2R5C*). *LASS4*, *LRRC4*, and *PPP2R5C* have been shown to be hypermethylated in colorectal cancer.[1,2,3] The Cologuard Plus procedure incorporates bisulfite conversion of non-methylated cytosine residues to uracil in the DNA sequence to enable sensitive detection of hypermethylated *LASS4*, *LRRC4*, and *PPP2R5C*.

The second category of biomarker is non-DNA based and detects hemoglobin, which can be associated with colonic bleeding. Results from the molecular and hemoglobin assays are integrated by the Exact Sciences Analysis Software to determine a Positive or Negative result or an Invalid result.

3 Principles of the Procedure

The Cologuard Plus test is designed to analyze a patient's stool for the presence of DNA and hemoglobin markers which may indicate the presence of CRC or APL. Patients use the Cologuard Plus Collection Kit, consisting of a Container for collection of stool for DNA testing and a separate sampler (Tube) for collection of stool for hemoglobin testing. Both of these stool samples are required to obtain a Cologuard Plus test result.

In the processing procedure for DNA testing, the stool sample is mixed with buffer in the Container using the Sample Mixer. An aliquot of the buffered stool sample is centrifuged to pellet solids and generate supernatant. The assay procedure begins with treatment of the supernatant with an Inhibitor Removal Tablet to remove inhibitors that may affect the detection of the DNA biomarkers. Treated supernatant is then combined with denaturing reagents and incubated with target-specific magnetic particles using the Capture Incubator instrument to capture sequences for *LASS4*, *LRRC4*, *PPP2R5C* and *ZDHHC1* (reference gene).

Using automated processes for capture aspiration and Hamilton Microlab® STARlet (STARlet) instruments, targeted sequences are separated from the solution, washed, and eluted from the particles. Eluted DNA is treated with bisulfite conversion reagents and further purified with silica-coated magnetic beads from which DNA is eluted.

The Long-probe Quantitative Amplified Signal (LQAS) technology combines real-time PCR and invasive cleavage to perform allele-specific amplification and detection of methylated target DNA in the Molecular Assay. Purified DNA is mixed with the LQAS reaction master mix and processed using a real-time cycler. Each marker is monitored separately through an independent fluorescence detection channel.

In a parallel workflow, the Hemoglobin Assay stool sample is prepared and analyzed in a quantitative Enzyme-Linked Immunosorbent Assay (ELISA) that determines the concentration of hemoglobin in the sample. Each sample is added to a single well of a 96-well deep well plate (DWP) and combined with magnetic capture beads pre-coupled with anti-hemoglobin antibody, and then washed to remove any unbound material. A second anti-hemoglobin antibody conjugated to the enzyme horseradish peroxidase (HRP) is then added to the wells and incubated with a colorimetric substrate for HRP. After the reaction is stopped and the absorbance is read on a plate reader, the level of hemoglobin present in the stool sample is calculated using a calibration curve prepared from a set of calibrators with known hemoglobin concentrations.

Run control samples for both the Molecular Assay and the Hemoglobin Assay are tested along with patient samples to show that the process has been performed appropriately. CG2 DNA Controls and Hb Bead Based Controls are required in each run to obtain valid assay results. Results from the molecular and hemoglobin assays are integrated by the Exact Sciences Analysis Software to determine a Positive or Negative reportable result or an Invalid result.

Individual results could be marked as invalid for multiple reasons, including:

- An error occurred during processing on the automated platform.
- Background data collected during the LQAS PCR run was above the allowable limit.
- *ZDHHC1* concentration was below the limit of 2.4 log strands.
- A sample was user-invalidated within the software due to known operator manual processing error.

In the event of an invalid test, up to 2 re-tests may be performed.

4 Contraindications

The Cologuard Plus test is not indicated for use in patients who have the following:

- A personal history of CRC or APLs.
- A positive result from another CRC screening method within the last 6 months, or:
  - 12 months for a fecal occult blood test (FOBT) or a fecal immunochemical test (FIT)
  - 36 months for a FIT-DNA test
- A family history of CRC, defined as having a first-degree relative (parent, sibling, or child) with a CRC diagnosis at any age.
- Personal history of any of the following high-risk conditions for CRC:
  - A diagnosis of Inflammatory Bowel Disease (Chronic Ulcerative Colitis, Crohn’s Disease).
  - A diagnosis of a relevant familial (hereditary) cancer syndrome or other polyposis syndrome, including but not limited to: Familial adenomatous polyposis (FAP or Gardner’s), Hereditary non-polyposis colorectal cancer syndrome (HNPCC or Lynch), Peutz-Jeghers, MYH-Associated Polyposis (MAP), Turcot’s (or Crail’s), Cowden’s, Juvenile Polyposis, Cronkhite-Canada, Neurofibromatosis, or Serrated Polyposis.

5 Warnings and Precautions

- Patients should not provide a sample if they are experiencing diarrhea or have known blood in their urine or stool (e.g., from bleeding hemorrhoids, bleeding cuts or wounds on their hands, rectal bleeding, or menstrual bleeding). Unexpected bleeding should be discussed with your healthcare provider.
- Reference national guidelines for the recommended screening ages for colorectal cancer.[4] The decision to screen persons over the age of 75 should be made on an individualized basis in consultation with your healthcare provider. Cologuard Plus test results should be interpreted with caution in older patients as the rate of false positive results increases with age.
- The Cologuard Plus test may produce false negative or false positive results. A false positive result occurs when the Cologuard Plus test produces a positive result, even though a colonoscopy will not find CRC or APL. A false negative result occurs when the Cologuard Plus test does not detect an APL or CRC even when a colonoscopy identifies either of these findings.
  - Out of 100 patients testing positive, approximately 3 patients will have CRC, 34 patients will have APL, 33 will have a non-advanced adenoma, and 30 will have no neoplastic findings.
  - Out of every 10,000 patients testing negative, approximately 2 will be falsely reassured that they do not have CRC. Out of every 100 patients testing negative, approximately 7 patients will be falsely reassured that they do not have APL.
- A negative Cologuard Plus test result does not guarantee the absence of cancer or advanced precancerous lesions. Patients with a negative Cologuard Plus test result should continue participating in colorectal cancer screening programs at the appropriate guideline recommended intervals.
- The performance of the Cologuard Plus test has been established in a cross-sectional study (i.e., single point in time). Programmatic performance of the Cologuard Plus test (i.e., benefits and risks with repeated testing over an established period of time) has not been studied. Non-inferiority or superiority of the Cologuard Plus test's programmatic sensitivity as compared to other recommended screening methods for CRC and APL has not been established.
- To ensure the integrity of the sample, the laboratory must receive the patient specimens within 144 hours of collection. Patients should send stool samples to the laboratory according to the instructions included in the Cologuard Plus Collection Kit.
- Read and understand the Safety Data Sheets (SDSs) for the reagents before storing, handling, or working with any chemical or hazardous material. SDSs are available by contacting Technical Services (refer to Contact Information or contact the original reagent manufacturer for other materials for guidance on storage, safe handling, disposal). Fecal samples should be treated as if they are potentially infectious.

6 Chemical Hazards

Hazardous ingredients used in the process depicted in this document are described in this section, with the associated hazard and precautionary statements. Hazard statements describe the type of hazard that may occur, while precautionary statements are recommendations to minimize or prevent effects described in the hazard statements. Pictograms, the signal word (i.e., DANGER), and hazard statement identifier(s) are on the component labels. Refer to the Safety Data Sheets (SDS) for additional information.

Read and understand the Safety Data Sheets (SDSs) before storing, handling, or working with any hazardous material.

WARNING: Do not use sodium hypochlorite (bleach) to decontaminate surfaces or dispose of waste from steps using Bisulfite Solution (CG2 BIS SLN) or Capture Solution (CAP SLN). Salts from these reagents are not compatible with cleaning products containing bleach.

6.1 Hazard and Precautionary Statements

6.1.1 Capture Solution

Component	Hazard and Precautionary Statements
200670 	CAP SLN, Capture Solution • H302: Harmful if swallowed. • H314: Causes severe skin burns and eye damage. • H318: Causes serious eye damage. • P260: Do not breathe mist, vapors, spray.

Component	Hazard and Precautionary Statements
 DANGER	• P264: Wash hands, forearms, and face thoroughly after handling. • P270: Do not eat, drink or smoke when using this product. • P280: Wear eye protection, protective clothing, protective gloves. • P301+P312: If swallowed: Call a poison center or doctor if you feel unwell. • P301+P330+P331: If swallowed: Rinse mouth. Do NOT induce vomiting. • P303+P361+P353: IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower. • P304+P340: IF INHALED: Remove person to fresh air and keep comfortable for breathing. • P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. • P310: Immediately call poison center/doctor. • P330: Rinse mouth. • P363: Wash contaminated clothing before reuse. • P405: Store locked up. • P501: Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation.

6.1.2 CG2 Stop Solution

Component	Hazard and Precautionary Statements
201640  WARNING	CG2 STP SLN, CG2 Stop Solution • H317: May cause an allergic skin reaction. • P261: Avoid breathing mist/vapors/spray. • P272: Contaminated work clothing should not be allowed out of the workplace. • P280: Wear protective gloves, protective clothing, chemical goggles, and face protection. • P302+P352: If on skin: Wash with plenty of water. • P333+P313: If skin irritation or a rash occurs: Get medical advice/attention. • P363: Wash contaminated clothing before reuse. • P501: Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation.

6.1.3 CG2 Bisulfite Conversion Solution

Component	Hazard and Precautionary Statements
201643  DANGER	CG2 BIS SLN, CG2 Bisulfite Conversion Solution • H314: Causes severe skin burns and eye damage. • P260: Do not breathe mist, vapors, spray. • P264: Wash hands, forearms, and face thoroughly after handling. • P280: Wear eye protection, protective clothing, protective gloves. • P301+P330+P331: If swallowed: rinse mouth. Do NOT induce vomiting. • P303+P361+P353: IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower • P304+P340: IF INHALED: Remove person to fresh air and keep comfortable for breathing. • P305+P351+P338: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. • P310: Immediately call poison center/doctor. • P363: Wash contaminated clothing before reuse. • P405: Store locked up.

Component	Hazard and Precautionary Statements
	• P501: Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation.

6.1.4 CG2 Binding Solution

Component	Hazard and Precautionary Statements
201644  WARNING	CG2 BND SLN, CG2 Binding Solution • H302: Harmful if swallowed. • H315: Causes skin irritation. • H319: Causes serious eye irritation. • P264: Wash hands, forearms, and face thoroughly after handling. • P270: Do not eat, drink or smoke when using this product. • P280: Wear eye protection, protective clothing, protective gloves. • P301+P312: If swallowed: Call a POISON CENTER or doctor if you feel unwell. • P302+P352: IF ON SKIN: Wash with plenty of soap and water. • P305+P351+P338 - IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. • P330: Rinse mouth. • P332+P313: If skin irritation occurs: Get medical advice/attention. • P337+P313: If eye irritation persists: Get medical advice/attention. • P362: Take off contaminated clothing. • P501: Dispose of contents/container to hazardous or special waste collection point, in accordance with local, regional, national and/or international regulation.

7 Materials Required

7.1 Reagents

NOTE: Distribution of the CG2 DNA and Hemoglobin Bead Based Assay components is restricted to Exact Sciences Laboratories.

Reagents in the following tables are listed either according to the Supplemental Lot Information Barcode (SLIB) group they are a part of, or to the ancillary group. SLIBs are imported into the Analysis Server Software which enables the test to be performed using the set of reagents in the SLIB with the Cologuard 2 Test Definition and the Exact Sciences System Software v2.2.

The SLIB contains:

- an assigned central part number heading the component part numbers of all the reagents in that SLIB group.
- an assigned central lot number heading the reagent lot numbers of all the reagents in that SLIB group.
- the assigned values for calibrators, expected ranges for calibrator results, and required results for controls in valid runs, including result call and/or acceptance ranges.

Essentially, reagents in a SLIB group form a virtual kit and must be used together for appropriate performance. The Analysis Server Software enforces these master-lot groupings. However, controls in SLIB groups, even though are matched by their lots, are not required to be used together.

A SLIB cover page accompanies a SLIB and it contains a catalog number (REF) to which a Unique Device Identifier is assigned. It lists the part numbers of the packs in which reagents comprising a SLIB group are transported to the Laboratory. Pack labels have pack part numbers, total number of reagent containers (bottle/tubes) and the volume contained in these containers. Each pack part number is related to the component part number of each of the reagents in the SLIB group. The maximum number of tests that can be performed using reagents in a SLIB with a particular central lot number are also indicated on the cover page.

Included in the table below are the Master Barcode PNs for reagents, which are part of the 1D barcode on reagent bottles and are coded into the system software to ensure that reagent lots are matched.

NOTE: *The Cologuard Plus test reagent labels may include CG2 in the nomenclature.*

7.1.1 DNA and LQAS Reagents

Table 7-1: Reagents in the DNA and LQAS SLIB Group

REF 100729	CG2 DNA and LQAS Reagents Supplemental Lot Information			Σ 750K	
Component PN	**Master Barcode PN**	**Pack PN**	**Description**	**Amount per vessel**	**Storage Temperature**
201356	201356	100690	CG2 DEN SLN, CG2 Denaturation Solution • 0.16 M NaOH solution	1 × 7 mL	15°C–30°C
201643  DANGER	201358	100688	CG2 BIS SLN, CG2 Bisulfite Conversion Solution • Ammonium bisulfite solution • Protect container from light.	1 × 1 L	2°C–8°C
201637	201361	100687	CG2 DES SLN, CG2 Desulphonation Solution • 0.1 M NaOH solution • Prepare working solution before use according to instructions in The Cologuard Plus Test Laboratory Procedure section.	1 × 800 mL	15°C–30°C
201774	201363	100689	CG2 BND BDS, CG2 Binding Beads • Magnetic silica particles	1 × 450 mL	15°C–30°C
201366	201366	100686	CG2 CAR SLN, CG2 Carrier Solution • Bovine Serum Albumin, Tris, EDTA • Reagent may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.	1 × 7 mL	-25°C–-15°C
201369	201369	100675	CG2 ELU BFR, CG2 Elution Buffer • Tris, EDTA Solution • Reagent may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.	1 × 9 mL	-25°C–-15°C
201372	201372	100675	CG2 MIX, CG2 Oligo Mix • Oligonucleotides, FRET probes, dNTPs • Store protected from light. • Reagent may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.	1 × 2.4 mL	-25°C–-15°C
201376	201376	100675	CG2 ENZ, CG2 Enzyme Mix • Enzymes in a buffer with glycerol • Reagent may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.	1 × 190 µL	-25°C–-15°C

REF 100729	CG2 DNA and LQAS Reagents Supplemental Lot Information			Σ 750K	
Component PN	**Master Barcode PN**	**Pack PN**	**Description**	**Amount per vessel**	**Storage Temperature**
201379	201379	100675	CG2 D CAL 1, CG2 DNA Calibrator 1, High • Target DNA in buffer with carrier DNA • Reagent may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.	1 × 110 µL	-15°C -25°C
201382	201382	100675	CG2 D CAL 2, CG2 DNA Calibrator 2, Mid • Target DNA in buffer with carrier DNA • Reagent may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.	1 × 110 µL	-15°C -25°C
201385	201385	100675	CG2 D CAL 3, CG2 DNA Calibrator 3, Low • Target DNA in buffer with carrier DNA • Reagent may be shipped at 2 to 8°C. Transfer to -25 to -15°C upon receipt.	1 × 110 µL	-15°C -25°C

7.1.2 DNA Controls

Table 7-2: Reagents in the DNA Controls SLIB Group

REF 100728	CG2 DNA Controls Supplemental Lot Information			Σ 690K	
Component PN/	**Master Bar Code PN**	**Pack PN**	**Description**	**Amount per vessel**	**Storage Temperature**
201734	201388	100671	CG2 D CTRL 1, CG2 DNA Control 1, High • Oligonucleotides, Tris, EDTA with Carrier DNA	1 × 800 mL	8°C 2°C
201735	201391	100672	CG2 D CTRL 2, CG2 DNA Control 2, Low • Oligonucleotides, Tris, EDTA with Carrier DNA	1 × 800 mL	8°C 2°C
201736	201394	100673	CG2 D CTRL 3, CG2 DNA Control 3, Negative • Oligonucleotides, Tris, EDTA with Carrier DNA	1 × 800 mL	8°C 2°C
201737	201482	100674	CG2 D CTRL 4, CG2 DNA Control 4, NTC • Tris, EDTA with Carrier DNA	1 × 800 mL	8°C 2°C

7.1.3 Hemoglobin Bead Based Assay Reagents

Table 7-3: Reagents in the Hemoglobin Bead Based Assay SLIB Group

REF 100727	CG2 Hemoglobin Bead Based Assay Reagent Supplemental Lot Information			Σ 375K	
Component PN/	**Master Barcode PN**	**Pack PN**	**Description**	**Amount per vessel**	**Storage Temperature**
201919	201327	100691	CG2 BEAD, CG2 Hb Capture Beads • Mouse anti-Human Hemoglobin Antibody coated Magnetic Beads • Equilibrate to room temperature before use.	1 × 400 mL	2°C–8°C
201638	201330	100692	CG2 CONJ, CG2 Antibody Conjugate • Mouse anti-Human Hemoglobin Antibody-HRP Conjugate • Protect container from light.	1 × 800 mL	2°C–8°C
201639	201332	100693	CG2 SUBS, CG2 Substrate • Tetramethylbenzidine in buffer • Protect container from light.	1 × 800 mL	2°C–8°C
201640  WARNING	201334	100694	CG2 STP SLN, CG2 Stop Solution • Acidic Buffered Solution	1 × 800 mL	2°C–8°C
201336	201336	100695	CG2 CAL, CG2 Hemoglobin Assay Calibrator • Human Hemoglobin, buffer (lyophilized) • Reconstitute and equilibrate to room temperature before use.	1 each	2°C–8°C

7.1.4 Hb Bead Based Controls

Table 7-4: Reagents in Hb Bead Based Controls SLIB Group

REF 100730	CG2 Hb Bead Based Controls Supplemental Lot Information			Σ 675K	
Component PN	**Master Barcode PN**	**Pack PN**	**Description**	**Amount per vessel**	**Storage Temperature**
201338	201338	100696	CG2 CTRL 1, CG2 Hemoglobin Control 1, High • Human Hemoglobin, buffer (lyophilized) • Reconstitute and equilibrate to room temperature before use.	1 each	2°C–8°C

REF 100730	CG2 Hb Bead Based Controls Supplemental Lot Information	Σ 675K

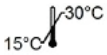
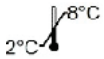
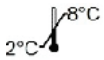
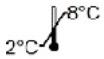
Component PN	Master Barcode PN	Pack PN	Description	Amount per vessel	Storage Temperature
201340	201340	100697	CG2 CTRL 2, CG2 Hemoglobin Control 2, Low • Human Hemoglobin, buffer (lyophilized) • Reconstitute and equilibrate to room temperature before use.	1 each	2°C–8°C
201342	201342	100698	CG2 CTRL 3, CG2 Hemoglobin Control 3, Negative • Buffer (lyophilized) • Reconstitute and equilibrate to room temperature before use.	1 each	2°C–8°C

7.1.5 Ancillary Materials

Ancillary materials are provided separately.

Table 7-5: Ancillary Reagents

Component PN	Master Barcode PN	Orderable PN	Description	Amount per Container	Storage Temperature
100543	100502	100543	CG2 CNV WSH, CG2 Conversion Wash Concentrate • Tris Buffer • Prepare working solution before use according to instructions in The Cologuard Plus Test Laboratory Procedure	1 × 900 mL	15°C–30°C
200204 200631 200895	N/A	N/A	STL BFR, Stool Buffer • Tris, EDTA Solution	1 × 20 L 1 × 1,000 L 1 × 15 kL	15°C–30°C
200151	N/A	100285	TABLT, Inhibitor Removal Tablet • (PVPP) Polyvinylpolypyrrolidone with excipient	95 each	15°C–30°C
201645	200122	100518	CG2 CAP WSH, CG2 Capture Wash • MOPS Buffer, NaCl	1 × 20 L	15°C–30°C
100627	N/A	100627	FILT, Spin Filter • Spin filters for 50 mL tubes	1 Bag of 300	15°C–30°C
200670 	N/A	100251	CAP SLN, Capture Solution • Guanidine Thiocyanate • If precipitate is observed, heat at 35 to 50°C to solubilize.	1 × 4 L	15°C–30°C

Component PN	Master Barcode PN	Orderable PN	Description	Amount per Container	Storage Temperature
 DANGER					
201644  WARNING	201406	100700	CG2 BND SLN, CG2 Binding Solution • Guanidine Hydrochloride with citrate buffer • If precipitate is observed, heat at 35 to 50°C to solubilize.	1 × 10 L	
100588	100503	100588	CG2 WSH, CG2 Hb Bead Based Assay Wash • Phosphate Buffer with detergent	1 × 800 mL	
100542	201346	100542	CG2 REC BFR, CG2 Reconstitution Buffer • 10% Albumin in Tris buffered solution containing an antimicrobial agent.	1 × 800 mL	
201920	201353	100699	CG2 CAP BDS, CG2 Capture Beads • Magnetic particles with covalently bound oligonucleotide probes	1 × 400 mL	

7.2 Exact Sciences System Software

The Cologuard 2 Test Definition with the Exact Sciences System Software v2.2 is comprised of a suite of interacting software applications and is used to perform the assay processes that are described within this IFU. The software applications are:

- Exact Sciences STARlet Interface Software
- Exact Sciences Reader Control Software
- Exact Sciences Cycler Control Software
- Exact Sciences Analysis Software

Refer to LBL-10438 *Exact Sciences System Software v2.2 User's Manual for Use with Cologuard 2.0* for further details on the software and the applications. Refer to LBL-10439 *Exact Sciences Cycler Control Software v2.2 User's Manual* for description of the Cycler Control Software application.

7.3 Procedural Warnings and Precautions

Warnings and notes emphasize important reagent information and critical instructions for safely performing laboratory procedures.

- Use standard laboratory precautions in accordance with applicable federal, state, and local regulations.
- Personal protective equipment should be worn for protection against hazardous materials.
- Laboratory areas should be cleaned and maintained according to good laboratory practices for clinical laboratories processing biological specimens.
- Refer to user's manuals for decontamination procedures for instruments and equipment.
- Product components (residual product, packaging, waste) may be considered laboratory waste. Dispose in accordance with applicable federal, state, and local regulations.

8 Instruments

8.1 Instrumentation

The instruments that are part of the Cologuard Plus System and required to perform the laboratory procedure for the Cologuard Plus test are listed in the table below. These instruments and supporting software are provided and installed separately through Exact Sciences service prior to training of laboratory personnel. The Cologuard Plus Test Laboratory Procedure section outlines the specific use of these instruments for performing the Cologuard Plus test assay.

Ensure that instrumentation is maintained per the original manufacturers' recommendations for safe use of these instruments; refer to the original manufacturers' user manuals. For instruments manufactured by Exact Sciences, refer to the following manuals.

- LBL-10008 Exact Sciences Sample Mixer 2 User's Manual
- LBL-10012 Exact Sciences Capture Incubator 2 User's Manual
- LBL-0102 Exact Sciences Capture Aspirator User's Manual

Table 8-1: Instruments Required for the Cologuard Plus Test Assay

Instrument	Manufacturer/Supplier	Part #
Sample Mixer 2 (120V) or equivalent	Exact Sciences	200441
Solaris™ 2000 Open Air Orbital Shaker or equivalent	Thermo Fisher Scientific or equivalent	09034202 or equivalent
Tube Shaker, Base 50 mL or equivalent	Exact Sciences	200481
Tube Shaker, Rack 50 mL or equivalent	Exact Sciences	200483
Capture Shaker Rack or equivalent	Exact Sciences	300551
Capture Incubator 2	Exact Sciences	100212
Capture Incubator Tube Lift	Exact Sciences	300547 200485
Capture Aspirator or equivalent • Vacuum Trap Box Kit • Vacuum Pump (optional)	Exact Sciences	300490 • 200529 • 200225
STARlet	Hamilton	66654-01
HBB STARlet	Hamilton	66654-02
Epoch2 Integration Kit	Hamilton	97370-01
QuantStudio™ 5 Dx Real-Time PCR System	Thermo Fisher Scientific	A47326 or A47327
BioTek® Epoch™ 2 Absorbance Microplate Reader, Exact Sciences Configuration	Agilent Technologies	EP-OCH2NSXCT
System Computer	Exact Sciences	200822 or equivalent

8.1.1 Custom Dye Calibrations Required for Use of QS5 Dx Instruments

Custom dye calibrations are required for use of QS5 Dx instruments for the Cologuard Plus test assay. Calibration instructions are captured in the QS5 DX User manual, *QuantStudio™ 5 Dx Real-Time PCR Instrument User Guide, Catalog Number A47324, Publication Number 100091230.* Required dyes and naming convention are in the table below.

Table 8-2: Custom Dye Calibration Plates

Description	Calibration Name (Instrument and Software)
FAM Tmer Dye Calibration Plates	FAM TE
HEX Tmer Dye Calibration Plates	HEX TE
CAL Fluor Red 610 Tmer Dye Calibration Plates	CFR610 TE
Quasar 670 Tmer Dye Calibration Plates	QUA670 TE

8.2 Instrument Warnings and Precautions

- Users should familiarize themselves with detailed user information contained in equipment user manuals prior to following the Cologuard Plus Laboratory Procedure section.
- CAUTION: To ensure safety, users should not override the door lock mechanism, attempt to open a running instrument unless prompted to do so by the software, or reach into a running instrument.
- Conduct instrument maintenance according to individual instrument user's manuals on all instruments to ensure safe and appropriate use.

- Conduct STARlet daily and weekly maintenance as required. Failure to empty liquid waste may result in release of hazardous materials into the environment. Failure to empty tip waste may result in an aborted run and/or contamination of the instrument deck.
- Ensure that the tips match the tip tray type on the STARlet. Do not consolidate tips into trays that do not match the tip tray type.
- Load only full trays of clean tips with no gaps, or an invalid run may result.

8.3 Materials Required But Not Provided

The quantity of each item required should be designed to meet the laboratory's workflow. All reagents and instruments are qualified for IVD use under the Exact Sciences Quality System.

Table 8-3: Materials Required But Not Provided

Description	Manufacturer/Supplier	Part #
Centrifuge tubes with flip cap: Polypropylene tube, non-pyrogenic, DNA/RNA free, leak proof: 50 mL	Corning or equivalent	431789 or equivalent
Centrifuge tubes without screw cap: 50 mL	Corning / Fisher Scientific	431689 / 07-201-440
Centristar screw cap: 50 mL	Corning / Fisher Scientific	431688 / 07-201-439
Conductive filter tips in frames • 50 µL • 1000 µL	Hamilton	 • 235948 • 235905
Deep Well Plates 96-well polypropylene, conical, skirted	Axygen / Fisher Scientific	P-DW-20-C / 14-222-353
Isopropyl Alcohol: ≥ 99.5% isopropanol, ≤ 0.2% water content	Various	Various
Pipette Tips Thermo Scientific™ ART™ Non-Filtered Extended-Length Wide-Bore Genomic pipette Tips: 200 µL	Thermo Fisher Scientific or equivalent	21-402-158
Plates • 96-well barcoded plate • MicroAmp™ Optical 96-well reaction plate with barcode: 0.2 mL	 • Corning / Fisher Scientific • Applied Biosystems / Fisher Scientific	 • 9065BC / NC1400562 • 4306737
Plate Seal • MicroAMP™ Optical Plate Seal or equivalent • PCR Plate Sealer, Sorenson BioScience or equivalent	 • Applied Biosystems/Fisher Scientific or equivalent • VWR or equivalent	 • 4311971 or equivalent • 14230-062 or equivalent
Reagent Reservoirs • Self-standing with lid: 60 mL • Self-standing with lid: 200 mL	 • Hamilton • Hamilton	 • 56694-01 • 56695-01
Screw Cap Microcentrifuge Tube Caps	Thermo Fisher Scientific	3471S
Tubes • Micro tubes, No Cap, Knurl, Skirted: 2 mL • Tube Screw cap: 5 mL • Micro tubes, No Cap, Knurl, Skirted: 2 mL	 • Sarstedt • Sarstedt • Thermo Fisher Scientific	 • 72.694.406 • 62.558.201 • 3490S
Trough Lid		

Description	Manufacturer/Supplier	Part #
• 50 mL • 200 mL	• Proto Labs Inc • Proto Labs Inc	• MOLD #45849 • MOLD #45060

9 Specimen Collection and Preparation for Analysis

Specimens for use with the Cologuard Plus test should be collected with the Cologuard Plus Collection Kit, including a stool sample for DNA testing (Container) and a stool sample for Hemoglobin testing (Tube). Detailed instructions for sample receipt and processing are outlined in The Cologuard Plus Test Laboratory Procedure below.

- Stool samples should be received in the sample container from the Cologuard Plus Collection Kit.
- The incoming Cologuard Plus Collection Kit should not be stored above 30°C.
- Detailed instructions regarding Receipt of the Cologuard Plus Collection Kit are outlined in The Cologuard Plus Test Laboratory Procedure section.
- Samples may be stored by the laboratory until processing. Detailed processing instructions are outlined in The Cologuard Plus Test Laboratory Procedure section.

10 The Cologuard Plus Test Laboratory Procedure

10.1 Assay Overview

The assay procedure includes steps for DNA Capture, DNA Preparation, LQAS Assay, Hemoglobin Bead Based Assay, and Data Analysis using the Exact Sciences System Software. DNA Capture steps can be processed in sets of up to 23 patient samples and 1 control sample. DNA Preparation and LQAS Assay steps are performed using the Microlab® STARlet (STARlet), custom built for Exact Sciences, and are processed in batches of up to 86 patient samples and the required controls. Hemoglobin Bead Based Assay steps are also performed on the STARlet in batches of up to 86 patient samples and required calibrators and controls. Optimal usage of Cologuard Plus reagents is achieved with four sets of DNA Capture, one full batch of DNA Preparation and LQAS Assay, and one full batch for the Hemoglobin Bead Based Assay. Test samples may be stored and run in maximum batch sizes to maximize utility of the reagents.

Controls are supplied in the DNA Control Kit and the Hb Bead Based Control Kit. Controls CG2 D CTRL 1, CG2 D CTRL 2, CG2 D CTRL 3, and CG2 D CTRL 4 are required for each batch of DNA Preparation and LQAS assay samples processed on the STARlet. One control is required for every distinct set of DNA Capture tubes. Hemoglobin Bead Based Assay Controls (CG2 CTRL 1, CG2 CTRL 2, and CG2 CTRL 3) are required for each batch of Hemoglobin Bead Based Assay samples processed on the STARlet.

10.2 Receipt of the Cologuard Plus Collection Kit

The patient collects a stool sample using the Cologuard Plus Collection Kit. Stool samples are sent to the laboratory according to the instructions that accompany the Cologuard Plus Collection Kit. Laboratory processing begins with receipt of the collection kit and preparation of stool for DNA capture.

1. Check that both the Tube (hemoglobin sample) and Container (DNA sample) are present. Check the Date of Collection and Time of Collection on the patient labels. Confirm that the specimens have been received within the valid collection window of 144 hours. Remove the samples and discard packaging in accordance with local regulations.
2. Mix the hemoglobin sample tube to dislodge stool from collection probe barbs, e.g., by vortexing for 20 seconds.
3. Store the samples appropriately until processing:
   a. The hemoglobin sample can be tested for up to 14 days from receipt if stored at 2 to 8°C.
   b. The DNA sample can be stored at 15 to 30°C or at 2 to 8°C and should be processed within 8 days of collection.

NOTE: *The barcoded identification number on the hemoglobin sample and DNA sample scanned by the STARlet at setup for DNA 1 Beads to DWP and Hemoglobin Bead must match exactly for assay results to be matched into an overall Cologuard Plus test result.*

NOTE: *In order to ensure successful automated scans, the barcodes affixed to the hemoglobin sample and the DNA sample must follow the appropriate barcode format, resolution, placement, and ANSI/ISO specifications as directed by the STARlet instrument manual. The hemoglobin sample tube will use a 1-dimensional barcode; stool homogenate tubes will use a 2-dimensional barcode.*

10.3 Preparation of Stool Homogenate for DNA Testing

1. Weigh the Container and record the Container Weight.
2. Calculate Stool Weight.
   - Calculated stool weight = Measured Container Weight (g) – 535 g (empty container + preservative weight).
3. Based on the calculated Stool Weight, adjust the stool:buffer ratio as follows (See Table 10-1) below:
   - If calculated stool weight is less than or equal to 0 g, sample is invalid and cannot be tested.
   - If calculated stool weight is greater than 0 g and less than or equal to 72 g, proceed to Step 4.
   - If calculated stool weight is greater than 72 g and less than 300 g, calculate amount of Stool Buffer to add. Stool Buffer can be added by volume with a tolerance limit of ±5 mL **or** by weight (see table below). Open the Container and add the Stool Buffer. Proceed to Step 4.
   - If stool weight is greater than or equal to 300 g, the sample is invalid and cannot be tested.
4. Close the Container lid following instructions in the Sample Mixer User's Manual.
5. Place the Container in the Sample Mixer, secure the container, and close the mixer door. Initiate the mixing cycle.
6. When the mixing is complete, remove the Container from the mixer, and then remove the lid from the Container.
7. Prepare 50 mL tubes with barcoded labels to identify the sample.
   NOTE: *50 mL tubes with flip caps (Corning or equivalent) are only to be used with stool homogenates, all subsequent steps in the process must use 50 mL tubes with screw caps.*

   NOTE: *It is recommended to prepare more than one 50 mL tube per sample to allow for repeat testing if necessary.*
8. Transfer up to 45 mL of homogenate sample to at least two 50 mL tubes per sample if possible.
   NOTE: *Avoid liquid volumes over 45 mL, as this could result in broken tubes or burst caps due to freezing expansion.*
9. Freeze samples at -15°C or colder for at least 8 hours or ensure they are completely frozen prior to use. Discard any remaining homogenate and the Container according to local regulations. Homogenate samples may be stored long term at <-15°C for a month, or at -80°C for up to 11 years. Tube (hemoglobin) samples may be stored long term at -80°C for up to 11 years.

Table 10-1: Adjust Stool:Buffer Ratio Based on Calculated Stool Weight

Stool Weight (X)	Stool Buffer to Add*		Additional Information
	By Volume (mL)	By Weight (g)	
X ≤ 0g	N/A	N/A	Invalid sample
0 g < X ≤ 72 g	N/A	N/A	Sample adequately buffered
72 g < X ≤ 280 g	4X – 290	1.04(4X – 290)	Dilution yields 1 g stool per 4 mL buffer
280 g < X < 300 g	(1,143 – X)/1.04	1,143 – X	Dilution maximized based on capacity of Container
X ≥ 300 g	N/A	N/A	Invalid Sample
*Stool Buffer may be added by volume or by weight. The density of the buffer (1.04 g/mL) is used in conversions between volume and weight.			

10.4 DNA Capture

10.4.1 Prepare and Label Sample Tubes

1. Remove frozen stool aliquot samples from storage.
   - Thaw samples at 2 to 8°C until the next processing steps. Do not leave at 2 to 8°C for more than 87 hours. Alternatively, thaw samples at 15 to 30°C for up to 6 hours.
2. Remove CG2 DNA controls (CG2 D CTRL 1-4) from storage.
3. Create unique barcoded labels for clean 50 mL conical tubes for each on-test sample and control. The barcodes for the controls must match those on the container. These barcode labeled tubes will be used in the following steps which include:
   a. addition of Inhibitor Removal Tablet (denoted as "TAB" or equivalent), and

b. use of Spin Filter (denoted as “CAP” or equivalent).

NOTE: *Do NOT use 50 mL tubes with flip caps.*

10.4.2 Prepare Supernatant

1. Centrifuge the thawed stool sample aliquots for 45 minutes at 4500 × g. Ensure that the centrifuge is balanced.
2. When the centrifugation is complete, carefully remove the tubes.
   NOTE: *If the interface between pellet and supernatant becomes noticeably disrupted (e.g., tube is dropped or inverted), repeat the previous two steps.*
3. Confirm that the labels from the centrifuged stool sample aliquots and DNA control tubes match the labels on the prepared, clean tubes for the next step.
4. Add one Inhibitor Removal Tablet to each barcode-labeled “TAB” tube before transferring samples.
5. Transfer 20 mL of supernatant from the centrifuged sample into the respectively labeled “TAB” tube.
   NOTE: *Transfer supernatant without disturbing the solid/liquid interface. Avoid transferring any material from the pellet, or any material floating on the surface of the supernatant.*
   - If the total supernatant yield is between 1 mL and 20 mL, transfer the available volume to the “TAB” tube and bring the total volume to 20 mL with Stool Buffer.

   NOTE: *The used 50 mL tubes with stool pellet and excess supernatant may be discarded according to local regulations.*
6. Mix each of the CG2 DNA control bottles to homogenize, e.g., by inverting at least 4 times.
   NOTE: *The bottles of CG2 DNA Controls are stable for 3 months on opening.*
7. Transfer 20 mL of each CG2 DNA Control into the respectively labeled “TAB” tube.
8. Secure all “TAB” tubes with clean caps.
9. Transfer the capped tubes to the Capture Shaker Rack and orbital shaker and mix for 15 minutes at 400 RPM.
10. After mixing sample supernatant with the Inhibitor Removal Tablet, confirm that the labels match the labels on the prepared, clean “CAP” tubes for the next step.
11. Place one Spin Filter into each “CAP” labeled tube before transferring samples from Step 10.
12. Agitate each tube from Step 10 to suspend contents. Foam may be present in the tube after shaking; take care while pouring contents into the spin filter of the respectively labeled “CAP” tube. Do not over fill spin filter and leave sufficient space to close cap without splashing. Close the lid of the spin filter.

    NOTE: *The used “TAB” tube and cap may be discarded according to local regulations.*
13. Place spin filter tubes into the centrifuge. Ensure the centrifuge is balanced and spin for 6 minutes at 3300 × g.
14. Remove the tubes from the centrifuge. For each tube, remove the spin filter from the tube.

    NOTE: *In the event of a broken spin filter, remove and discard filter, cap the sample tube, and vortex the filtrate. Repeat steps 10-14.*

    NOTE: *The used filter may be discarded according to local regulations.*
15. Determine volume of clarified supernatant in the tube.
    a. If a tube contains less than 5 mL, discard the sample.
    b. If a tube contains greater than or equal to 5mL and less than 17 mL, bring volume to 17 mL with Stool Buffer.
    c. If a tube contains more than 17 mL, adjust to 17 mL by removing excess clarified supernatant.
16. Proceed to the next step within 6 hours or store at 2 to 8°C for up to 6 days.

10.4.3 DNA Capture Incubation

NOTE: *The clarified supernatant is stable at room temperature for up to 6 hours. If the tubes of prepared clarified supernatant were stored at 2 to 8°C, equilibrate at 15 to 30°C for 30 minutes. These are the "capture tubes" described in this section.*

NOTE: *Ensure each capture set includes at least one DNA control.*

1. Remove CG2 Capture Beads from storage.

   NOTE: *CG2 Capture Beads must be used within 28 hours of being placed at room temperature.*

   NOTE: *CG2 Capture beads are stable for 3 months after opening.*
2. Power on Capture Incubator 2 and select “EXAS CG2 v1.0.1.0". Press play to preheat the incubator.

   NOTE: *Preheating will take approximately 15 minutes.*
3. Inspect Capture Solution for precipitate. If precipitate is present, warm at 35 to 50°C until solubilized. Invert to mix after warming.

4. Mix CG2 Capture Beads until homogeneous on a bottle roller set at four revolutions every 42-62 seconds for at least two hours to suspend the beads. Beads must be added to samples within 20 minutes of mix ending.
5. Add 125 µL of the homogenized CG2 Capture Beads to each of the capture tubes.
6. Add 13 mL of Capture Solution to each capture tube and then secure with a clean cap.
7. Place all capture tubes into the Capture Incubator 2 using the Capture Incubator Tube Lift, place tubes filled with approximately 30 mL water into each empty position, close the lid, and press play on the preheated incubator. Once the samples have been inserted into the preheated incubator, the program must be started within 1 minute.
   NOTE: *Confirm the Capture Incubator 2 successfully preheated before inserting capture tubes.*

   NOTE: *The capture incubation will take approximately 1 hour and 23 minutes.*

   NOTE: *If the Capture Incubator 2 produces an error message while running the method, refer to the Capture Incubator 2 User's Manual. A description of each error code displayed on the Capture Incubator 2 can be found in the Appendices.*
8. When the method reaches completion, remove capture tubes from the Capture Incubator 2, remove and discard caps, and place open tubes in the Capture Aspirator.
   NOTE: *In the Capture Aspirator, empty positions in rows that contain sample (capture) tubes should be occupied to ensure optimal aspiration. Place a 50 mL conical tube filled with approximately 30 mL of water into each empty space within a row occupied by a sample.*
9. Perform capture aspiration using the "BIND 10" method.
   NOTE: *If the Capture Aspirator produces an error message or if there is a power outage while running the aspirator, refer to the Capture Aspirator User's Manual (Troubleshooting section).*
10. Remove the capture tubes from the Capture Aspirator and inspect for complete aspiration.
    NOTE: *If incomplete aspiration is observed, bring tube volume to 10 mL using Capture Wash, mix by pulse vortexing and repeat the "BIND 10" method.*
11. Add 750 µL of CG2 Capture Wash to each tube.
12. Mix tubes to suspend and disperse the capture beads in the capture wash. Shake tubes at 400 RPM for 1 minute using the Capture Shaker rack and orbital shaker or an equivalent means of mixing.
    NOTE: *If beads are not suspended, repeat mixing until suspended.*
13. Remove the tubes; close with a clean cap and store at 2 to 8°C if not proceeding with Captured Sample Transfer. Closed tubes containing capture wash and beads can be stored for up to 4 days before transfer. Repeat step 12 for all samples stored at 2 to 8°C prior to proceeding with Captured Sample Transfer.
    NOTE: *If beads are not transferred from the tubes to the DWP in the next steps within three hours, repeat step 12 to ensure bead dispersion.*

10.4.4 Captured Sample Transfer

NOTE: *A full batch of samples (up to 86 patient samples plus 4 controls) requires four sets of DNA Capture. If fewer than 86 patient samples are processed, all 4 DNA controls are required.*

1. Prepare STARlet for use.
   a. Log into the Exact Sciences STARlet Interface Software.
   b. Under the Maintenance Monitor, check to see whether the daily, weekly, and monthly are highlighted green. If all three are green, maintenance does not need to be performed. The monitor also lists the last date checked.
   c. If daily, weekly, and/or monthly are highlighted red, maintenance needs to be performed. Perform required weekly maintenance before daily maintenance. Select the required maintenance type that is red, select the green "Run" to begin, and follow the software prompts to perform the maintenance.

   NOTE: *Monthly maintenance does not cover daily or weekly maintenance. Weekly maintenance covers all of the daily maintenance tasks. If weekly maintenance is being executed, daily maintenance does not need to be run.*

   NOTE: *Cologuard 2.0 methods will not begin if required maintenance has not been completed successfully.*

   NOTE: *Ensure the tip and liquid waste containers contain sufficient room before starting a run.*
2. Select Cologuard 2.0 and the "DNA 1 Beads to DWP" setup run on the Exact Sciences STARlet Interface Software, then select 'Load Setup' to initiate the run.

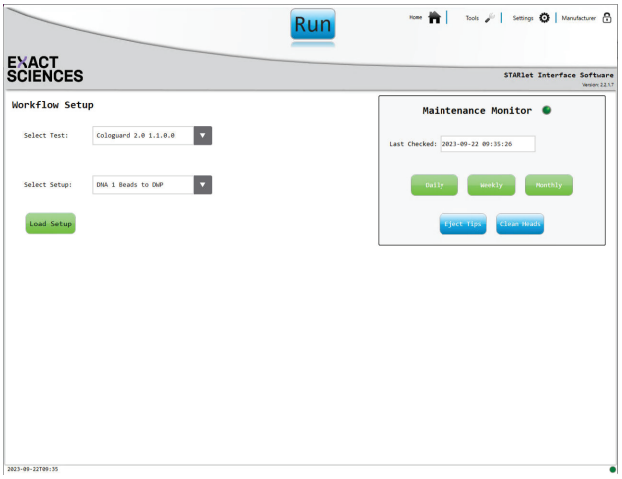


Figure 10-1: Cologuard 2.0 and DNA 1 Beads to DWP setup

3. The deck layout diagram appears as shown below. Load the appropriate carriers on the loading tray according to the deck layout.

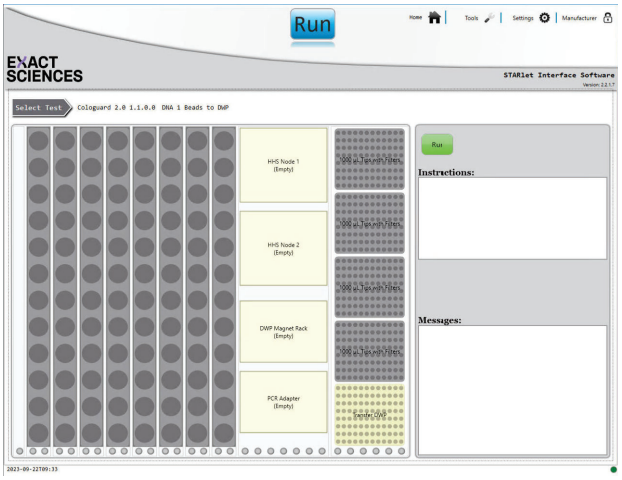


Figure 10-2: Deck Layout Diagram

4. Load a unique barcode-labeled DWP such that the barcode label is only on the right side of the DWP to ensure the code is scanned by the method. Load four full trays of 1,000 µL CO-RE II tips in the right-hand tip carrier with no gaps in the trays, or an invalid run may result.
5. Load and fully seat uncapped samples and control tubes into 50 mL Tube Carriers.
   NOTE: *Confirm sufficient volume of liquid in each tube by visual inspection before loading into carriers. If liquid is not present, do not load tubes into the carrier.*
   a. Place uncapped samples and control tubes in the sample carriers, moving from back to front and left to right, with no empty positions between tubes.
   NOTE: *All four controls (CG2 D CTRL 1, CG2 D CTRL 2, CG2 D CTRL 3, and CG2 D CTRL 4) must be present within the sample carriers for the run to proceed.*

   NOTE: *Do not use expired reagents as it will result in invalid assay results and is only used for trouble shooting when appropriate user privileges are applied.*
   b. Place all sample carriers on deck, including empty carriers. Ensure that the barcode is visible in the carrier slot on the right.
   NOTE: *Empty positions are not permitted in sample carriers, except after the last loaded sample. Ensure there are no empty positions between samples. Load all carriers regardless of the number of samples, placing empty carriers at the end (right).*

   NOTE: *Carriers with unread sample barcodes will be unloaded. The barcode positioning must be adjusted, and the carrier reloaded until the barcode is successfully rescanned, or barcode sample IDs may be entered manually by the operator.*

   NOTE: *The DNA Controls loaded in the run are checked to match lot numbers from active DNA Control Kit SLIBs imported into the Analysis software (see Procedural Notes and Precautions for directions to enter Supplemental Lot Information). A run will not proceed without one of each type of control from an active DNA Control Kit SLIB.*
6. Select the green ‘Run’ button to start the loading process.
7. When all the carriers have been properly loaded, select ‘Next’.
8. A prompt will open on the right side of the screen which will have a default of 90 samples and controls. If this is the correct number of samples for the run, proceed by selecting the green arrow. If the run has less than 90 tubes, the new sample amount can be entered. After entering the correct number of samples and controls for the particular run, select the green arrow button.
9. As carriers are loaded into the STARlet, a series of prompts may appear in the Instructions box, prompting the user to correct sample loading issues such as unread barcodes. Items with unread or incorrect barcodes are indicated in red on the deck layout graphic. Select ‘Next’ after each action is performed until the run starts.

NOTE: *Ensure response to any tip pick up error is within three hours. Beads must be transferred from the 50 mL conical tubes (sample tubes) to the DWP within three hours after vortex mixing. If there is no response for more than three hours, the beads will settle in the sample tubes, potentially invalidating the patient sample results.*

10. On completion of the run, select 'Next' to unload the carriers.
11. After captured sample transfer is complete, remove the sample carriers.
    NOTE: *The used capture tubes may be discarded according to local regulations.*
12. Samples in Captured Sample DWP (Transfer DWP) are stable at room temperature for up to 8 hours. If not proceeding to the DNA 2 DWP to Assay Plate method setup, cover the barcode-labeled Transfer DWP with a plate seal and store at 2 to 8°C until ready to setup the assay plate. Sealed Transfer DWPs containing capture wash and beads can be stored for up to 4 days before use.
13. The results for the 'DNA 1 Beads to DWP' setup run (valid or invalid by sample or control) can be viewed using Analysis Client. A run will be invalid if a required control transfer was invalid.

10.5 DNA Preparation and LQAS Assay

DNA Preparation and LQAS Assay steps are processed in batches of up to 86 samples from the DNA Capture steps. Input samples include up to 86 patient samples in addition to CG2 D CTRL 1, CG2 D CTRL 2, CG2 D CTRL 3, and CG2 D CTRL 4 in each batch. DNA preparation and the LQAS assay plate setup are performed on the STARlet. The DNA 2 DWP to Assay Plate method for the Exact Sciences STARlet Interface Software guides the operator through loading the samples, consumables, and reagents onto the system.

The system uses barcodes to identify samples, reagents, and controls. The barcodes on the samples and DWP are used to ensure tracking to the final result while the reagent barcode tracking ensures that the matched lots of reagents are used together and that the reagents have not expired.

DNA and LQAS Reagent Supplemental Lot Information, contained in a .slib file, is used to transfer lot and calibrator information into the Exact Sciences System Software. Similarly, CG2 DNA Controls Supplemental Lot Information is used for transfer of the control values and acceptance limits for the particular lots of controls used in the procedure. The information needs to be entered only once for each unique Supplemental Lot Information lot number.

Detailed instructions are provided in the software screens on the correct positioning of each reagent and all consumables, samples, and carriers. Calibrators for the assay (CG2 D CAL 1, CG2 D CAL 2 and CG2 D CAL 3) serve as validity controls for the LQAS runs. Patient samples, controls, and calibrators are set up in one 96-well LQAS reaction plate. Additional instructions can be found in LBL-10438 *Exact Sciences System Software v2.2 User's Manual for Use with Cologuard 2.0.*

The steps for Automated DNA Preparation and LQAS Plate Setup are completed in about 7 hours. After the DNA preparation steps are completed, the instrument prompts the user to mix, uncap, and place reagents for the LQAS plate setup. When the LQAS plate is ready to transfer to the thermocycler, the user removes the plate, covers with an optical plate seal, centrifuges to ensure the reagents are at the bottom of all wells, and runs the plate on the QS5 Dx Real-Time PCR System. The LQAS analytic run is completed in approximately 1.5 hours. Once complete, the data are exported to the Exact Sciences Analysis Software and the run results are calculated. Assay runs are considered valid if the calculated results from all DNA controls are within the expected ranges included in the CG2 DNA Controls Supplemental Lot Information, the calibration curve meets the acceptance criteria, and no fatal processing errors were detected by the system.

10.5.1 Automated DNA Preparation and LQAS Assay Setup

Reagent Preparation

1. Assemble the following reagents.
   Table 10-2: Reagents required for DNA Preparation

Part #	Component Abbreviation / Name
201645	CG2 CAP WSH, CG2 Capture Wash
201356	CG2 DEN SLN, CG2 Denaturation Solution
201643	CG2 BIS SLN, CG2 Bisulfite Conversion Solution
201637	CG2 DES SLN, CG2 Desulphonation Solution
201774	CG2 BND BDS, CG2 Binding Beads
201644	CG2 BND SLN, CG2 Binding Solution
100543	CG2 CNV WSH, CG2 Conversion Wash Concentrate

Part #	Component Abbreviation / Name
201366	CG2 CAR SLN, CG2 Carrier Solution

NOTE: *The following reagents are stable for 3 months after opening: CG2 Capture Wash, CG2 Binding Solution, CG2 Conversion Wash, CG2 Bisulfite Conversion Solution, CG2 Desulphonation Solution, and CG2 Binding Beads.*

2. Create a 30% CG2 Desulphonation Solution, 70% isopropanol solution. Mix to ensure solution is homogenized, e.g., by inverting at least 4 times.
3. Mix the CG2 Conversion Wash Concentrate to ensure it is homogenized e.g., by inverting at least 4 times. Once mixed, this concentrate need not be mixed again for up to 48 hours.
4. To prepare CG2 Conversion Wash, create a 25% CG2 Conversion Wash, 75 % isopropanol solution. Mix to ensure solution is homogenized e.g., by inverting at least 4 times.
5. Captured Sample DWP preparation:
   a. If the Transfer DWP was not stored at 2 to 8 °C, place directly onto the STARlet carrier as specified in the STARlet Setup section.
   b. If the Transfer DWP was sealed and stored, remove from storage. If not used immediately, the DWP may remain at room temperature for a maximum of 8 hours. Briefly centrifuge plate to bring liquid to bottom of the wells.
   c. Remove the seal prior to initiating the DNA 2 run.
6. Ensure the following reagents (appropriately master-lot matched) are available. They will be loaded in the LQAS Plate Setup step. These reagents are packaged for single use and the leftover reagents after the run cannot be reused.

Table 10-3: Ensure LQAS Plate Setup Reagents are Master-lot Matched

Part #	Component Abbreviation / Name
201369	CG2 ELU BFR, CG2 Elution Buffer
201372	CG2 MIX, CG2 Oligo Mix
201376	CG2 ENZ, CG2 Enzyme Mix
201379	CG2 D CAL 1, CG2 DNA Calibrator 1, High
201382	CG2 D CAL 2, CG2 DNA Calibrator 2, Mid
201385	CG2 D CAL 3, CG2 DNA Calibrator 3, Low

STARlet Setup

1. Log into the STARlet Interface Software.
2. Complete any required daily, weekly, or monthly maintenance on the STARlet. Refer to Captured Sample Transfer for further instruction.
3. If SLIBs have not been previously imported into the Analysis Software, follow instructions for Enter Supplemental Lot Information in the Procedural Notes and Precautions section.
4. Select Cologuard 2.0 and the “DNA 2 DWP to Assay Plate” setup run on the Exact Sciences STARlet Interface Software, then select ‘Load Setup’ to initiate the run.

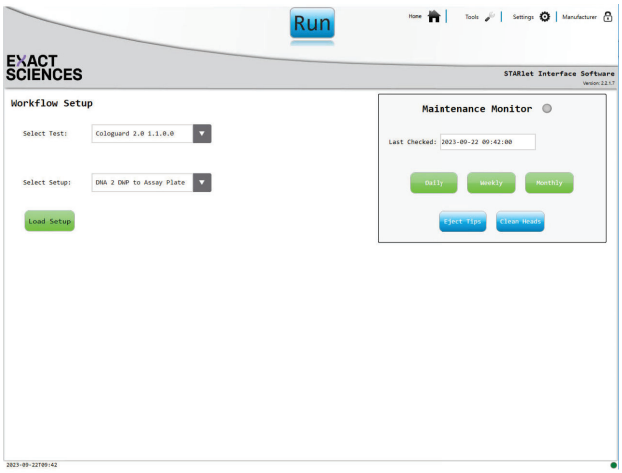


Figure 10-3: DNA 2 DWP to Assay Plate Setup

5. The deck layout diagram appears as shown in following figure. Select the green ‘Run’ button to start the loading process.

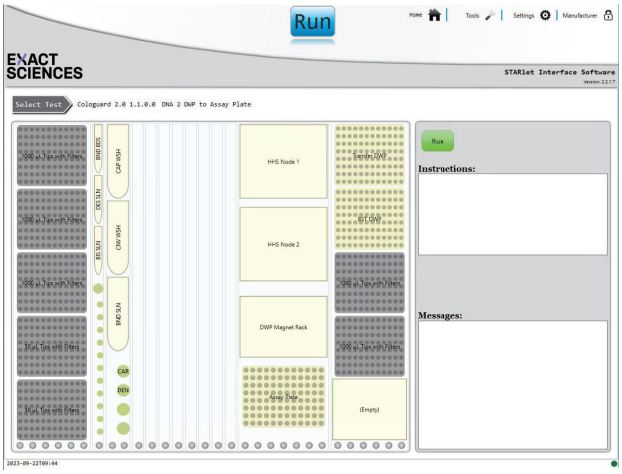


Figure 10-4: DNA 2 DWP to Assay Plate Layout Diagram

6. A prompt appears on the right side of the screen stating, “Place carriers on the autoload tray. Verify that the caps have been removed from CG2 CAR

SLN and CG2 DEN SLN and that the seal has been removed from the Transfer DWP. Press Next to continue after caps and seal have been removed."
NOTE: *Failure to remove caps will abort run and result in failure.*

NOTE: *Failure to remove seal from Transfer DWP before start of the run will result in the run aborting due to a hardware error and may cause damage to the pipetting channels.*

7. Load the appropriate carriers on the loading tray according to the deck layout.
   a. Load the Transfer DWP in the back position above the right-hand tip carrier and remove the seal if one is present. In the position below, place a clean, unique barcode-labeled DWP (BST DWP) for conversion/cleanup. Ensure the barcode is only on the right side of the BST DWP.
   b. Place two full trays of clean 1,000 µL CO-RE II tips in the right-hand tip carrier. Ensure that there are no gaps in the trays, or this may result in an invalid run result. Any tips present in this carrier from a previous run must be presumed to be used and discarded to prevent possible aborted runs or cross-contamination of samples.

NOTE: *Ensure the tip and liquid waste containers contain sufficient room before starting a run.*

8. Load one MicroAmp 96-well plate, 0.2 mL with a unique barcode to the front as indicated by the deck layout.
9. Create barcoded labels for troughs into which reagents will be poured which match the barcodes on the reagent primary container labels. Apply barcodes to the appropriate troughs on the loading tray. Load the barcoded reagent troughs as shown in the on-screen deck layout into the indicated carrier positions.
   a. If a SLIB needs to be entered to continue setup of the run, import the SLIB into the Analysis Client Software prior to selecting 'Next' to reload the carrier.
   b. See Table 10-4 for components and special instructions for individual reagents.

NOTE: *Reagents are loaded and checked to match lot numbers from a SLIB imported into the Analysis software. Reagents are identified and confirmed to be the correct lot and location by scanning their barcodes as they are loaded on the instrument. Users should not run with master lot mismatch errors, except for troubleshooting purposes when appropriate user privileges are applied.*

NOTE: *If any reagents are not recognized or do not match to a SLIB in the system, the carrier containing the mismatched reagents is unloaded. The software prompts the user to correct the issue and then select 'Next" to reload the carrier.*

NOTE: *It is critical the correct reagent volume, as stated in the table below, is added to the deck to ensure proper liquid handling by the STARlet. The STARlet may abort the run if liquid level in a reagent trough is insufficient.*

NOTE: *Create and apply barcodes to troughs so that barcode is placed on the curved edge of the trough, starting at the top of the trough with the curved edge of trough on the left, as shown in the following figure.*

NOTE: *The following reagents have a stability of 6 hours upon loading on the deck: CG2 Capture Wash, CG2 Denaturation Solution, CG2 Bisulfite Conversion Solution, Prepared CG2 Desulphonation Solution, CG2 Binding Beads, CG2 Binding Solution, Prepared CG2 Conversion Wash, CG2 Carrier Solution, and CG2 Elution Buffer.*

Figure 10-5: Placing Barcode on Trough

Table 10-4: Reagents Loaded on the STARlet

Part #	Component	Additional Instructions
201645	CG2 CAP WSH, CG2 Capture Wash	Create and apply a barcode to a clean 200 mL trough. Mix until homogenous by shaking container at 100 RPM on Max Q 4000 (or equivalent) for 60 seconds , then transfer 170 mL to the trough within 48 hours of mixing. Trough may be re-used if rinsed with distilled water and allowed to dry.
201356	CG2 DEN SLN, CG2 Denatura-tion Solution	Mix until homogenous, e.g., by inverting at least 4 times,

Part #	Component	Additional Instructions
		then UNCAP and place vial in the indicated carrier.
201643	CG2 BIS SLN, CG2 Bisulfite Conversion Solution	Create and apply a barcode to a clean 50 mL trough. Mix until homogenous, e.g., by inverting bottle at least 4 times, then transfer 17 mL to the trough within 48 hours of mixing. The trough may be re-used if rinsed with distilled water and allowed to dry.
201637	CG2 DES SLN, CG2 Desulphonation Solution (after isopropanol addition)	Create and apply a barcode to a clean 50 mL trough. Transfer 29 mL to the trough and cover with 50 mL Trough Lid. The trough may be re-used if rinsed with distilled water and allowed to dry.
201774	CG2 BND BDS, CG2 Binding Beads	Create and apply a barcode to a clean 50 mL trough. Mix until suspended, e.g., by inverting bottle at least 70 times, then immediately transfer 9 mL to trough. The trough may be re-used if rinsed with distilled water and allowed to dry.
201644	CG2 BND SLN, CG2 Binding Solution	Create and apply a barcode to a clean 200 mL trough. Mix until homogenized, e.g., by shaking container at 100 RPM on Max Q 4000 (or equivalent) for 60 seconds, then transfer 100 mL to the trough within 48 hours of mixing. The trough may be re-used if rinsed with distilled water and allowed to dry.
100543	CG2 CNV WSH, CG2 Conversion Wash (after isopropanol addition)	Create and apply a barcode to a clean 200 mL trough. Transfer 200 mL to the trough and cover with 200 mL Trough Lid. The trough may be re-used if rinsed with distilled water and allowed to dry.
201366	CG2 CAR SLN, CG2 Carrier Solution	UNCAP and place vial in the indicated carrier. CG2 CAR SLN can be placed on the deck frozen. The solution will thaw and be mixed on deck.

10. Load three full trays of 1,000 µL CO-RE II tips and two full trays of 50 µL CO-RE II tips into the left-hand tip carrier.

    NOTE: *Load only full trays of clean tips with no gaps, or an invalid run may result.*
11. When all carriers on the loading tray have been properly loaded with reagents, samples, and consumables, and caps have been removed from the CG2 CAR SLN and CG2 DEN SLN tubes, and the seal (if present) has been removed from the Transfer DWP, select 'Next'.

    NOTE: *Transfer DWPs should be processed only once by the STARlet.*
12. As carriers are loaded into the STARlet, a series of prompts may appear in the Instructions box, prompting the user to correct reagent loading issues such as mismatched lots or unread barcodes, if applicable. Items with unread or incorrect barcodes are indicated in red on the deck layout graphic. Select 'Next' after each action is performed.

DNA Preparation

1. After all required reagents, DWPs, and tips have been loaded and the LQAS plate barcode has been scanned, the automated method begins. The STARlet records the LQAS plate barcode as the run identifier.
2. During the method, liquid transfer verification is used by the software to monitor the transfer of reagents and samples.
3. The Messages box displays the status notifications during the run, such as approximate end time of incubation steps.

LQAS Plate Setup

NOTE: *Plan time and resources accordingly. Once the LQAS Plate Setup is complete (Steps 1-10 below), the run on the QS5 Dx instrument must be started within 60 minutes.*

1. Assemble LQAS Assay reagents listed in Step 6 of the Automated DNA Preparation and LQAS Assay Setup, Reagent Preparation section. Place reagents at room temperature for at least 30 minutes. Ensure reagents are fully thawed and have acclimated to room temperature.
2. Approximately 6 hours after the DNA 2 DWP to Assay Plate setup run begins, the carriers with reagents will be automatically unloaded for LQAS

Plate Setup, and a prompt will appear on the right side of the screen stating, “Place UNCAPPED reagent tubes in the indicated positions. Press Next to continue.”. The deck layout diagram will appear as shown below.

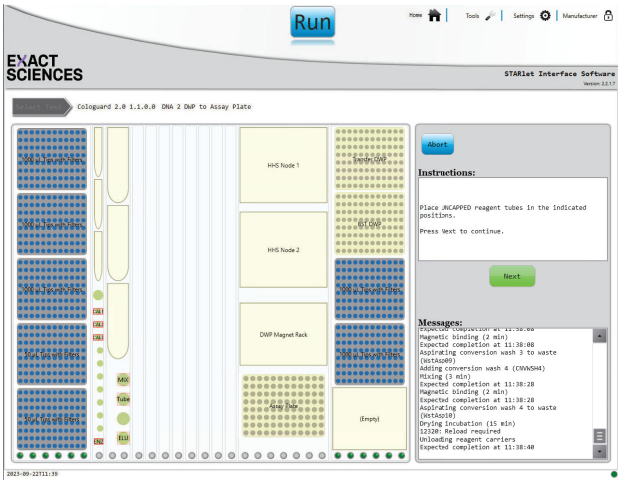


Figure 10-6: LQAS Deck Layout Diagram

3. Load reagents as shown in the on-screen deck layout into the indicated carrier positions.
   a. Remove used CG2 CAR SLN and CG2 DEN SLN tubes. Remaining non-LQAS reagents may also be removed if desired.
   b. See Table 10-5 for components and special instructions for individual reagents.
      NOTE: *Reagents are loaded and checked with reagents loaded at the start of the DNA 2 DWP to Assay Plate setup run to match lot numbers from a SLIB imported into the Analysis software. Reagents are identified and confirmed to be the correct lot and location by scanning their barcodes as they are loaded on the instrument. Users should not run with master lot mismatch errors, except for troubleshooting purposes when appropriate user privileges are applied.*

      NOTE: *Remove caps from reagents before loading for use.*

      NOTE: *If any reagents are not recognized or do not match to a SLIB in the Analysis Server, the carrier containing the mismatched reagents is unloaded. The software prompts the user to correct the issue and then select ‘Next’ to reload the carrier.*

      NOTE: *Barcodes on reagents are used to check expiry dates. Continuing a run with expired reagents will result in invalid assay results and can be used only for troubleshooting purposes when appropriate user privileges are applied.*

Table 10-5: LQAS Plate Setup Reagents

Part #	Component
201369	CG2 ELU BFR, CG2 Elution Buffer
201372	CG2 MIX, CG2 Oligo Mix
201376	CG2 ENZ, CG2 Enzyme Mix
201379	CG2 D CAL 1, CG2 DNA Calibrator 1, High
201382	CG2 D CAL 2, CG2 DNA Calibrator 2, Mid
201385	CG2 D CAL 3, CG2 DNA Calibrator 3, Low
Instructions for all components: Mix by vortexing for at least 5 seconds. Centrifuge briefly to ensure no bubbles are present and no liquid remains in the cap. Place the tube in the indicated carrier in the deck layout diagram.	

   c. Uncap and place an empty 5 mL tube in the indicated carrier.

      NOTE: *It is recommended to place barcodes on carrier rack positions where empty 5 mL tubes are expected to be placed.*

      NOTE: *Failure to remove caps will abort run and result in failure.*
4. When all carriers have been properly loaded with reagents and caps have been removed from all tubes, select ‘Next’.
5. As carriers are reloaded into the STARlet, a series of prompts may appear in the Instructions box, prompting the user to correct reagent loading issues such as mismatched lots or unread barcodes, if applicable. Items with unread or incorrect barcodes are indicated in red on the deck layout graphic. Select ‘Next’ after each action is performed.
6. After all required reagents have been loaded, the automated method resumes.
7. At the end of the run, the user is prompted to remove the 96-well LQAS assay plate and the BST DWP. Select ‘Next’ to unload carriers and remove the plates.
8. Select ‘OK’ to confirm removal of used consumables and reagents.
9. Seal the assay plate with an adhesive optical seal.

10. Briefly centrifuge the assay plate to bring the liquid to the bottom of the wells.

Run the LQAS Plate

1. Log into the Cycler Control Software.
2. Using a barcode scanner connected to the Cycler Control Software computer, scan the instrument barcode for a 'ready' and connected QS5 Dx IVD Real-Time PCR Instrument. After scanning the instrument, the tray door will open.
3. Load the assay plate into the instrument.
   NOTE: *The barcode for the assay plate must be facing outward when loaded on the instrument to ensure proper well alignment.*
4. Scan the assay plate barcode.
5. Scan the instrument barcode again. The tray door will close and the run will begin.
6. The background check method will run first. If the background check is successful, then the method will run to analyze the plate.
7. If the background check fails, a dialog will appear in the Cycler Control software indicating the wells that failed the check. The dialog includes an indication of the amount of time that has passed since the setup run completed to aid the user in determining if there is enough time to transfer the plate to another instrument.

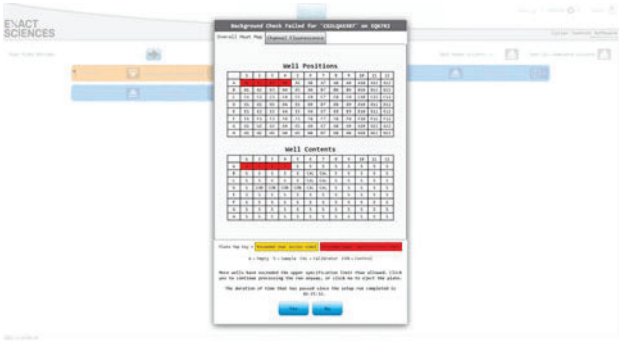


Figure 10-7: Cycler Control Software Dialog Box showing Background Check Failures

   a. Clicking the Yes button (or waiting 60 seconds for the dialog to clear automatically) will continue the run and analyze the plate.
   b. In this situation, the user can choose to analyze the plate or load the plate into another instrument.
8. Data from the analysis of the assay plate will automatically be uploaded to the Analysis Server.

10.6 Hemoglobin Bead Based Assay

The Hemoglobin Bead Based Assay is fully automated on the STARlet. The Exact Sciences STARlet Interface Software guides the operator through the loading of reagents, consumables, and samples. The system uses barcodes to identify samples and reagents. The barcode on each sample tube is used to ensure traceability to the final result.

The barcode on each reagent is used to identify the part number, the lot, and expiration date, and may be used to match lots of reagents as appropriate. Hemoglobin Bead Based Assay Reagents Supplemental Lot Information is used to transfer calibration and lot information into the Exact Sciences System Software. Similarly, the Hemoglobin Bead Based Control Kit Supplemental Lot Information is used to transfer the control values and acceptance limits for the particular kit lot used in the procedure.

The steps for Automated Hemoglobin Bead Based Plate Setup are completed in approximately 4 hours.

10.6.1 Automated Hemoglobin Bead Based Plate Setup

Prepare Samples

1. Remove the hemoglobin samples from storage.
2. Allow hemoglobin samples from 2 to 8°C storage to incubate at room temperature for 60 minutes prior to starting the automated method. Samples may remain at room temperature before method start for up to 24 hours.
3. Samples removed from -80°C storage should be incubated at room temperature until thawed and equilibrated to room temperature. Alternatively, these frozen samples can be thawed overnight at 4°C. Thawed samples should be vortexed for 5 seconds to mix.

Prepare STARlet

1. Log into the STARlet Interface Software.
2. Complete any required daily, weekly, or monthly maintenance on the STARlet.
3. If SLIBs have not been previously entered into the Analysis Software, follow instructions for Enter Supplemental Lot Information in the Procedural Notes and Precautions section.

Prepare Hemoglobin Bead Based Assay Reagents

1. Use following procedure for equilibrating reagents required for Hemoglobin Bead Based Assay:

a. The CG2 Reconstitution Buffer, CG2 Hb Bead Based Assay Wash, CG2 Stop Solution, CG2 Antibody Conjugate, and CG2 Substrate can be used straight from storage.

NOTE: *Protect CG2 Antibody Conjugate and CG2 Substrate from light.*

b. Remove CG2 Hb Capture Beads from 2 to 8°C storage.

c. Allow CG2 Hemoglobin Bead Based Controls and Calibrator to equilibrate at room temperature for 30 minutes after removing from storage.

d. Reconstitute the controls and Calibrator, and equilibrate at room temperature for another 30 minutes, for a total of 60 minutes as described below in Step 3.

Table 10-6: Reagents required for Hemoglobin Bead Based Assay

Part #	Component
201919	CG2 BEAD, CG2 Hb Capture Beads
201638	CG2 CONJ, CG2 Antibody Conjugate
201639	CG2 SUBS, CG2 Substrate
201640	CG2 STP SLN, CG2 Stop Solution
201336	CG2 CAL, CG2 Calibrator
201338	CG2 CTRL 1, CG2 Control 1, High
201340	CG2 CTRL 2, CG2 Control 2, Low
201342	CG2 CTRL 3, CG2 Control 3, Negative
100588	CG2 WSH, CG2 Hb Bead Based Assay Wash
100542	CG2 REC BFR, CG2 Reconstitution Buffer

NOTE: *The following reagents are stable for 3 months after opening: CG2 Hb Capture Beads, CG2 Antibody Conjugate, CG2 Substrate, CG2 Stop Solution, CG2 Hb Bead Based Assay Wash, and CG2 Reconstitution Buffer.*

2. Mix CG2 Hb Capture Beads until homogeneous using either a mechanical inverter set to 30 +/- 3 RPM for 15 minutes or a bench top bottle roller set to four revolutions every 42-62 seconds for 30 minutes. This can be performed at any point after removal from 2-8°C storage. Beads must be transferred within 15 minutes after mixing stops.
3. Invert CG2 Reconstitution Buffer at least 4 times prior to using for reconstitution. Approximately thirty minutes after equilibration begins, reconstitute the CG2 Calibrator, CG2 Control 1, CG2 Control 2, and CG2 Control 3 with 3 mL of CG2 Reconstitution Buffer each.

   NOTE: *Reconstituted and capped bottles of Hb Calibrator, CG2 Control 1, CG2 Control 2, and CG2 Control 3 are stable for up to 6 hours.*
4. Replace stoppers and invert to expose any lyophilized material present on the rubber stopper to the CG2 Reconstitution Buffer to ensure complete reconstitution.
5. Vortex reconstituted CG2 Calibrator and CG2 Controls at highest setting for at least 5 seconds.
6. Continue to equilibrate to the end of the 60-minute period.

STARlet Setup for Hemoglobin Bead Based Assay

1. Select Cologuard 2.0 and the “Hemoglobin Bead” setup run on the Exact Sciences STARlet Interface Software, then select ‘Load Setup’ to initiate the run as shown in the following figure.

Figure 10-8: Select Hemoglobin Bead Based Assay on the STARlet

2. The Hemoglobin Bead Based Assay deck layout appears on the screen. Select the green ‘Run’ button to start the loading process.

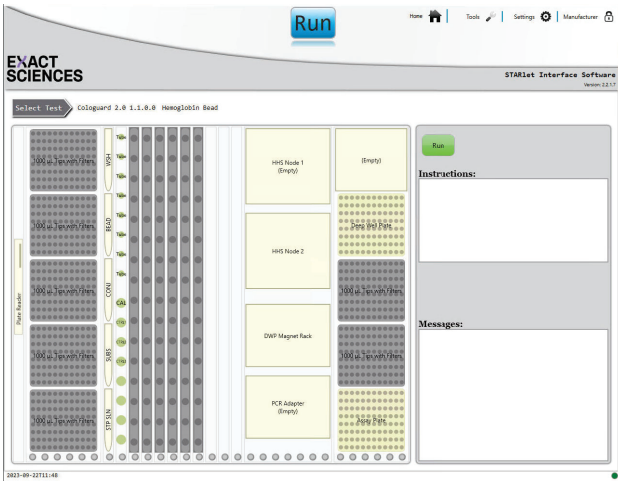


Figure 10-9: Hemoglobin Bead Based Assay Deck Layout

NOTE: *If the instrument does not detect a reader, a prompt will appear on the right side of the screen stating a reader is not connected or is not powered on. Select 'Yes' to continue setup or 'No' to retry the reader connection.*

NOTE: *If "Yes" is selected, plate will not be read automatically.*

NOTE: *If any carriers are present on the deck, a prompt appears to clear autoload tray, then select 'Next' to continue. Carriers will be unloaded.*

NOTE: *Verify the plate reader is empty, and place carriers on the autoload tray.*

NOTE: *Ensure the tip and liquid waste containers contain sufficient room before starting a run.*

3. Confirm sufficient volume of liquid in each sample Tube by visual inspection before loading into carriers. If liquid is not present, do not load the Tube into the carrier.
4. Place the sample Tubes with foil side up into the carriers working from the back of the STARlet to front and left to right.
   a. Orient the tubes ensuring that the barcodes are visible in the carrier slot.
   b. Push the tubes completely into the carrier positions.
   c. Empty positions are not permitted in sample carriers, except after the last sample tube. Ensure there are no empty spaces between samples. Load all six carriers regardless of number of samples, placing empty carriers to the right of loaded carriers.
   d. Carriers with unread sample barcodes will be unloaded. The barcodes must be adjusted, and the carrier reloaded until the barcode is successfully rescanned, or barcode sample IDs may be entered manually by the operator.

   NOTE: *Manually entered barcode sample IDs will have a 503 flag.*
5. Load reagents as shown in the on-screen deck layout into the indicated carrier positions. All the non-bead bulk reagents can be mixed by inverting 4 times and used for up to 48 hours without having to mix again prior to loading on the deck. See instructions in Table 10-7 for mixing CG2 Hb Capture Beads.

   NOTE: *It is critical that the correct reagent volume is added to the deck to ensure proper liquid handling by the STARlet. The STARlet may abort the run if liquid level in a reagent trough is insufficient.*

   NOTE: *Do not continue a run with expired reagents. It will result in invalid assay results and is only used for trouble shooting when appropriate user privileges are applied.*

   NOTE: *The following reagents are stable for 6 hours after being poured in troughs or on-deck: CG2 Hb Capture Beads, CG2 Antibody Conjugate, CG2 Substrate, CG2 Stop Solution, and CG2 Hb Bead Based Assay Wash.*
6. See the following table for components and special instructions for individual reagents.

   NOTE: *Create and apply barcodes to troughs so that barcode is placed in the middle of the trough along the top edge. Position the trough with the curved edge to the left, orient the barcode sticker with the top of the barcode aligned with the top of the trough as shown in the following figure.*

Figure 10-10: Place Barcode on Trough for Hemoglobin Assay

Table 10-7: Hemoglobin Assay Reagents to be Loaded on the Deck

Part #	Component	Additional Instructions
201919	CG2 BEAD, CG2 Hb Capture Beads	Create and apply a barcode to a clean 50 mL trough. Mix the bottle using either

Part #	Component	Additional Instructions
		a mechanical inverter set to 30 ± 3 RPM for 15 minutes, or a bench top bottle roller set to four revolutions every 42-62 seconds for 30 minutes, or an equivalent method of mixing. Transfer 12 mL to the trough within 15 minutes after mixing stops. The trough may be reused if rinsed with distilled water and allowed to dry.
201638	CG2 CONJ, CG2 Antibody Conjugate	Create and apply a barcode to a clean 50 mL trough. Mix by inversion at least 4 times, then transfer 12 mL to the trough. The trough may be re-used if rinsed with distilled water and allowed to dry after each use.
201639	CG2 SUBS, CG2 Substrate	Create and apply a barcode to a clean 50 mL trough. Mix by inversion at least 4 times, then transfer 12 mL to the trough. The trough may be re-used if rinsed with distilled water and allowed to dry after each use.
201640	CG2 STP SLN, CG2 Stop Solution	Create and apply a barcode to a clean 50 mL trough. Mix by inversion at least 4 times, then transfer 12 mL to the trough. The trough may be re-used if rinsed with distilled water and allowed to dry.
201336	CG2 CAL, CG2 Hemoglobin Assay Calibrator	Vortex at highest setting for 5 seconds, then UNCAP and place vial in the indicated carrier with the barcode showing through the open window.
201338	CG2 CTRL 1, CG2 Hemoglobin Control 1, High	Vortex at highest setting for 5 seconds, then UNCAP and place vial in the indicated carrier with the barcode showing through the open window.
201340	CG2 CTRL 2, CG2 Hemoglobin Control 2, Low	Vortex at highest setting for 5 seconds, then UNCAP and place vial in the indicated carrier with the barcode showing through the open window.
201342	CG2 CTRL 3, CG2 Hemoglobin Control 3, Negative	Vortex at highest setting for 5 seconds, then UNCAP and place vial in the indicated carrier with the barcode showing through the open window.
100588	CG2 WSH, CG2 Hb Bead Based Assay Wash	Create and apply a barcode to a clean 50 mL trough. Mix by inversion at least 4 times, then transfer 60 mL of assay wash to the trough. The trough may be re-used if rinsed with distilled water and allowed to dry after. **NOTE:** *Each CG2 WSH bottle has sufficient volume for at least 13 runs.*

7. Load 8 uncapped empty 2 mL tubes for calibrator dilution into the appropriate positions in the indicated carrier.

   NOTE: *It is recommended to place barcodes on carrier rack positions where empty 2 mL tubes are expected to be placed.*
8. Load two trays of 1,000 µL CO-RE II tips into the right-hand tip carrier.
9. Load five trays of 1,000 µL CO-RE II tips into the left-hand tip carrier.

   NOTE: *Load only full trays of clean tips with no gaps, or an invalid run may result.*

   NOTE: *Any tips present in the Wash Tips tray of the right-hand tip carrier (Position 3) from a previous run must be presumed to be used and discarded to prevent possible aborted runs or cross-contamination of samples.*
10. Load one unique barcode labeled 96-well plate with the barcode facing right.
11. Load one deep-well plate in the indicated position. Orient the 96-well deep-well plate so A1 is positioned in the top left corner of the carrier.
12. When all carriers are positioned on the autoload tray, address the prompt on the right side of the screen by selecting ‘Next’.
13. A prompt with a default of 86 samples will appear. If this is the correct number of samples for the run, select the green arrow to proceed. If the user has a different sample number from 86, enter the sample

count. After entering the correct number of samples for the run, select the green arrow. See figure below.

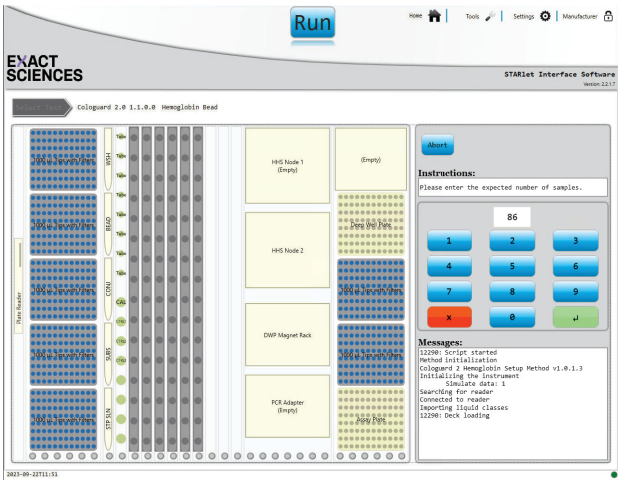


Figure 10-11: Hemoglobin Assay Loaded Deck

The STARlet will start the run by loading the carrier containing the 96-well barcoded plate and the deep-well plate. The barcode on the 96-well barcoded plate is read and recorded as the run identifier. The STARlet checks for the deep-well plate by using the iSWAP to pick up and replace the deep-well plate in its starting position.

The following system checks will occur at the onset of the run:

- The system will check for the correct location and lot (when applicable) of tips, reagents, or samples as they are loaded by scanning and recording the item barcodes.

  NOTE: *Items with unread or incorrect barcodes are indicated in red on the deck layout graphic. The software prompts the user to correct the issue and then select 'Next' to reload the carrier.*
- After scanning, the barcodes of the reagents and CG2 CAL are checked against the SLIB for master lot matching.

  NOTE: *Items with master lot mismatches are indicated in red on the deck layout graphic. The instrument will unload the carrier and the software prompts the user to correct the issue and then select 'Next' to reload the carrier.*
- The STARlet completes loading with the carrier containing five trays of 1,000 µL CO-RE II tips.

10.6.2 Automated Plate Processing

The STARlet will complete the setup and processing of the plate.

1. The user is prompted to unload the carriers and select 'Next' to acknowledge the unloading; this action will be recorded in the log file as the time the carriers were unloaded. STARet will not pause to wait for 'Next' to be selected and will continue with the run.
2. Prior to sample transfer to the 96-well barcoded plate, approximately 3.5 hours after the sample carriers are unloaded, the reagent and left-hand tip carriers are unloaded. After the plate is read and the run is complete, the right-hand tip carrier holding the plate is unloaded.

Select '**OK**' to complete the run when a dialog box appears stating the run is complete.

10.6.3 Manually Read Hemoglobin Plate

1. If the plate was not read automatically, the setup run was executed without a plate reader connected, or the connection to the plate reader was lost during the first read of the plate, check the plate ready time in the Messages box on the Run screen of the STARlet Interface Software. The plate can be read manually up to 45 minutes from the plate ready time.
2. Log into the Exact Sciences Reader Control Software. The Run screen appears as shown in figure below.

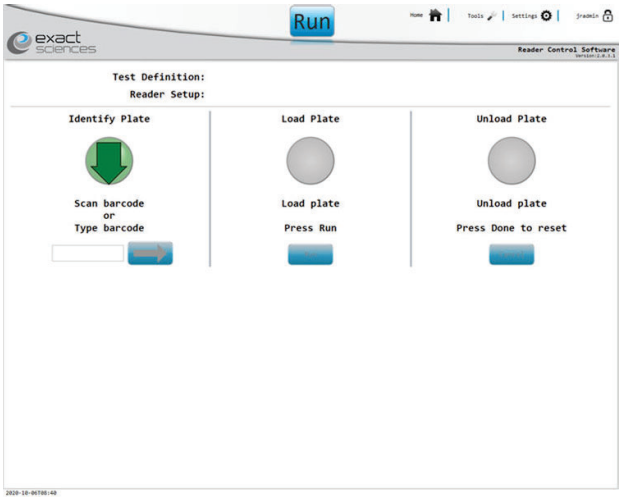


Figure 10-12: Run Screen for Manual Read of Hemoglobin Plate

3. Scan or enter the barcode of the Hemoglobin plate. For manual entry of barcode, repeat the barcode entry in the second field if required by site administrator setting. After typing the barcode(s), select the arrow button to continue.
4. Load plate into the Epoch™ 2 reader when prompted by the software.
   NOTE: *Place the plate into the reader so that embossed 'A1' is positioned in the back left corner*

of the reader tray. Incorrect placement will result in invalid results.

5. Press 'Run' to read the plate.
6. Unload the plate when prompted.
7. Press 'Done' to reset the software to be ready to read a new plate.
8. The assay data are automatically transmitted to the Analysis Server Software.
9. If a connection to the Exact Sciences Analysis Server Software is not available, data may be transferred manually. Copy the data file from C:\ExactSciences\Reader\Runs with file name <plate barcode>.<checksum>.reader and import into Analysis Software following instructions in LBL-10438 *Exact Sciences System Software v2.2 User's Manual for Use with Cologuard 2.0*.

10.6.4 Hemoglobin Sample Storage

1. When hemoglobin samples are unloaded during plate setup on the STARlet (approximately 30 minutes into a run with 86 samples), punctured Tubes can be stored with foil side up at 2 to 8°C for up to 14 days after date of receipt.
2. If repeat testing is required, see the Hemoglobin Sample Retesting section for detailed instructions.

10.6.5 Hemoglobin Sample Retesting

NOTE: *To avoid sample loss, use caution when handling the punctured tubes to avoid a sample spill.*

NOTE: *Ensure that the samples are run within 14 days after date of receipt.*

- If samples have not been punctured, place them at 2 to 8°C until ready to rerun in the Hemoglobin Bead setup.
- Samples may remain at room temperature for up to 24 hours. If samples have been punctured, rerun before the expiration of the allowed 24 hours at room temperature or store foil-side up at 2 to 8°C until ready to rerun the Hemoglobin Bead setup.
- Before rerunning, confirm that all sample tubes are correctly placed into sample racks. To rerun the setup, begin with Prepare Samples section.

11 Data Handling and Analysis

Data from the Molecular and Hemoglobin Bead Based Assays are integrated and analyzed by the Exact Sciences Analysis Server Software. The Exact Sciences Analysis Server Software maintains traceability of the sample to result through sample barcodes scanned during molecular and hemoglobin assay plate setup runs. For the molecular assay, data from the thermocycler (QuantStudio 5 Dx Real Time PCR System) are imported into the Exact Sciences Analysis Server Software and the fluorescence signal for each channel versus cycle time is analyzed to calculate a crossing point (Cp) where the detection threshold is exceeded. This value enables the calculation of detected concentration of each DNA marker using the respective calibrators. For the Hemoglobin Bead Based Assay, the absorbance data is imported from the reader and the hemoglobin concentration in each sample and control is calculated from the calibration curve.

The system uses the expected values and actual results of the calibrator and control samples to assign a run status (valid/invalid) for Molecular and Hemoglobin Bead Based Assay runs. Users review, comment upon, or invalidate sample run data in the software as required to capture any errors or invalid samples that occur during the assay procedure.

The software calculates an overall Cologuard Plus score for each sample by combining the released results of each marker for that sample (linked by sample ID). A Negative or Positive result is assigned for each sample based on the Cologuard Plus score. Invalid Cologuard Plus test results occur if any constituent assay results are invalid. Details on the use of the software can be found in LBL-10438 *Exact Sciences System Software v2.2 User's Manual for Use with Cologuard 2.0*.

11.1 Review and Release Molecular or Hemoglobin Assay Results

Once the assay runs are complete, the run data are imported into the Analysis Server Software and the assay run and individual sample assay results are calculated. Results of each assay may be reviewed in the Analysis Client Software before the software incorporates the results into the calculations that generate the Cologuard Plus test result.

1. Log into the Analysis Client Software.
2. The Runs List view displays a list of run(s) that have not been released. Apply filters to locate the run(s) to be released in the Runs table.

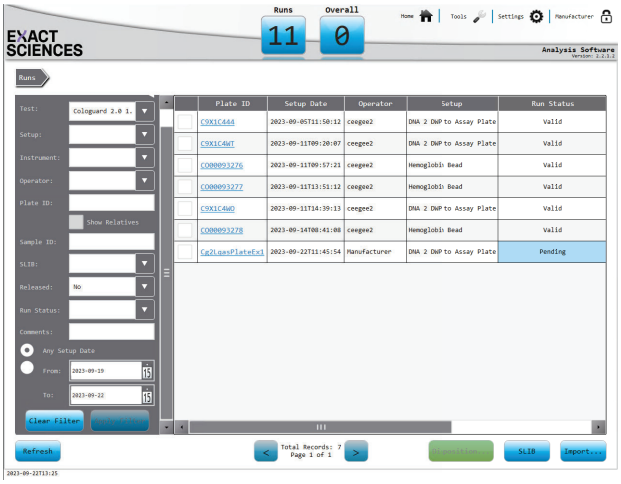


Figure 11-1: Runs List View in Analysis Client Software

NOTE: *To navigate back to the Runs List view at any time, select the 'Runs' button on the top of the screen. Filters will need to be re-applied.*

3. If run data files need to be manually imported (e.g., the computers running the other Exact Sciences System Software applications (STARlet Interface and Reader Control) are not connected to the computer running the Analysis Server Software), follow these steps:
   a. Select ‘Import’ on the bottom right of the screen to select a file to upload.
   b. Navigate to the folder containing the data and select the file to upload. Valid file types from STARlet Interface have .plate3 extensions, files from Cycler Control have .cycler2 extensions, and files from Reader Control have .reader extensions.
   NOTE: *Altering the file names may make files invalid for import.*
4. Once the run list includes the desired run, select the hyperlink under the Plate ID column to display Run details and select the triangle icon in the lower right corner of the Summary section to display all run information.

	Plate ID	Setup Date	Operator	Setup	Run S
	C9X1C444	2023-09-05T11:50:12	ceegee2	DNA 2 DWP to Assay Plate	Va
	C9X1C4WT	2023-09-11T09:20:07	ceegee2	DNA 2 DWP to Assay Plate	Va
	C000093276	2023-09-11T09:57:21	ceegee2	Hemoglobin Bead	Va
	C000093277	2023-09-11T13:51:12	ceegee2	Hemoglobin Bead	Va
	C9X1C4WO	2023-09-11T14:39:13	ceegee2	DNA 2 DWP to Assay Plate	Va

Figure 11-2: Selecting Hyperlink Under the Plate ID Column

5. The Run Detail view displays run information in the Summary section and on top tabs for Calibration, Calibrators, Controls, Samples, and Reagents with left side tabs for switching between assays in the same run.

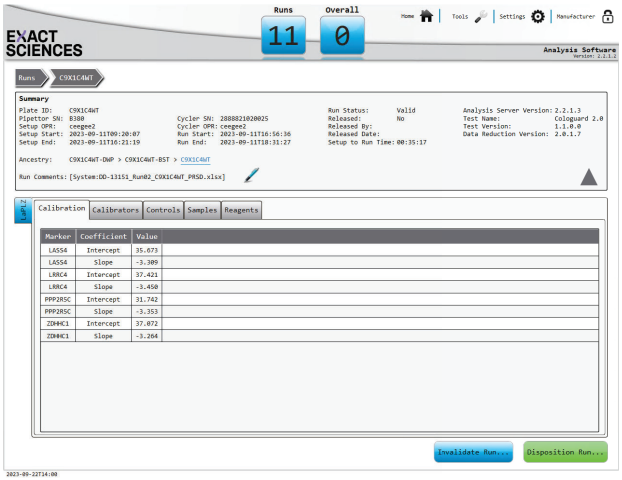


Figure 11-3: Run Detail View

6. Review data as desired in the Calibration, Calibrators, Controls, Samples, and Reagents tabs along the top of the data section.

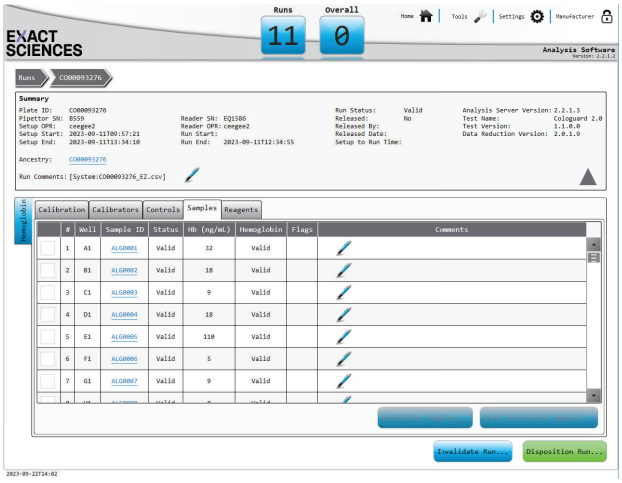


Figure 11-4: Review Data

   a. Each tab has specific information about the results of the testing for the displayed run. Select each tab to review the data.
   b. Plate IDs match the barcode on the 96-well plate used in the setup run.
   c. For the ‘Hemoglobin Bead’ setup run, one Plate ID is linked to one ‘Hemoglobin’ assay run in the Assay column.
   d. For the ‘DNA 2 DWP to Assay Plate’ setup run, one Plate ID is linked on one assay run in the Assay column.

e. The run status of the assays on a plate is marked as Pending until assay run data for the plate are uploaded from the Cycler Control Software, STARlet Interface Software with attached reader, or the Reader Control Software.

f. When an assay run status is either Valid or Invalid, the assay run results are ready for review.

7. After a setup run plate map is received from either a 'DNA 2 DWP to Assay Plate' or 'Hemoglobin' run, the Analysis Server Software exports a file listing samples with invalid results due to processing errors. This file may be useful for managing retest workflows. Details on this export can be found in *Invalid-to-Retest Setup Sample Export* section of the LBL-10438 *Exact Sciences System Software v2.2 User's Manual for Use with Cologuard.*

8. If user comment is desired, follow these steps.
   a. If user comment is desired for an individual control, calibrator, or patient sample, select the pen icon in the 'Comment' area for the sample. Enter the comment and select 'Apply' to save the comment.
   b. To comment on an entire run, select the pen icon in the 'Run Comments' field in the Summary section, enter the comment and select 'Apply' to save the comment.
   c. Each calibrator, control, and sample have a status listed as Valid or Invalid.

9. In the event of errors in manual processing steps or other errors observed, a user may invalidate sample results.

   NOTE: *Ensure that invalidation of individual sample results is completed prior to releasing the run.*

   a. To mark individual sample assay results as invalid, go to the Samples tab, select the checkbox for the individual sample(s), and select the 'Invalidate Samples...' button. Enter comment, username, and password to complete the action.

      NOTE: *Sample results invalidated by a user are permanently marked as invalid for that particular run once Run Disposition is performed and run data are Released or Closed. Invalidation/Undo Invalidation cannot be performed on Released or Closed sample results.*

   b. To mark all the results in a run as invalid, select 'Invalidate Run...' button. Press yes to confirm the invalidate action and enter username and password to complete the action.

      NOTE: *Runs invalidated by a user cannot be Released through a Run Disposition. Invalid assay results may only be Closed.*

10. To undo user invalidation prior to run disposition, (refer to Step 9.a above), select the checkbox for the individual sample and select on the 'Undo Invalidate...' button. Enter username and password to complete the action.

11. There are two ways to manually disposition a run. One way is within the Run Detail view for an individual assay plate, and the other is for dispositioning multiple runs at the same time within the Runs List view.
    a. To release an individual run of assay results within the Run Detail view, select the 'Disposition Run' button on the bottom of the screen and enter username and password to complete the action.
    b. To release multiple runs at the same time, go to the Runs List view displaying the list of runs that have not been released. To select a group of runs for action, enter a checkmark in the selection box for each run or mark the entire list by selecting the checkbox column header once to check all the boxes for the runs listed on the page (up to 100). Select the 'Disposition' button on the bottom of the screen and enter username and password to complete the action.

       NOTE: *Invalid or Pending runs cannot be Released, they may only be dispositioned as Closed.*

       NOTE: *Results from Closed runs are not available for overall test result interpretation.*

       NOTE: *Runs that have been Released or Closed cannot be invalidated by any user, nor can invalidation be undone on a Released or Closed run.*

12. Releasing an assay run makes the results available for calculation of the overall Cologuard Plus test result for the samples in the run.

11.2 Review and Release Overall Cologuard Plus Test Results

After users release valid 'DNA 2 DWP to Assay Plate' and 'Hemoglobin' run results, the software generates an overall Cologuard Plus score for each sample using each of the marker results for that sample and assigns a Negative or Positive result based on the Cologuard Plus score. Invalid Cologuard Plus test results occur if any of the constituent assay results are invalid.

If the Analysis Software is configured to disposition results automatically, then sample results will be released after Valid (Positive or Negative) or Invalid sample results have been generated. See the Exact Sciences System Software User's Manual for how to set this configuration option.

Alternatively, active users in the Supervisor or Administrator role may manually disposition (Release or Close) overall Cologuard Plus test results.

1. Log into the Exact Sciences Analysis Software.
2. Select the 'Overall' button to view the overall test results table. The filter defaults to show results that have not yet been released. Filters may be applied to narrow the table to show only specific samples or types of results.

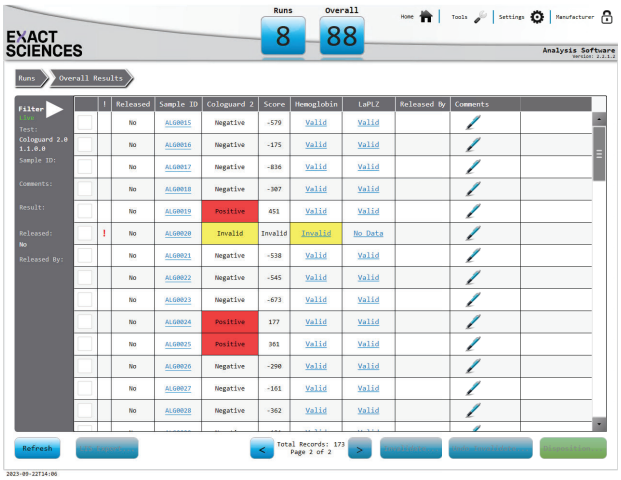


Figure 11-5: Viewing Overall Results in Exact Sciences Analysis Software

   a. For each sample, ! (Flags), Released, Sample ID, Cologuard Plus (Test Result), Score, and Hemoglobin, and Molecular assay results are displayed on the Overall Results view.
   b. To select a group of samples for action, enter a checkmark in the selection box of each sample in the group or select the checkbox column header in the Overall Results table to select all samples in the group.

3. To review a summary of individual assay results for a sample, select the hyperlink for the Sample ID. The Sample Detail view displays the overall result and individual assay results for the selected sample.

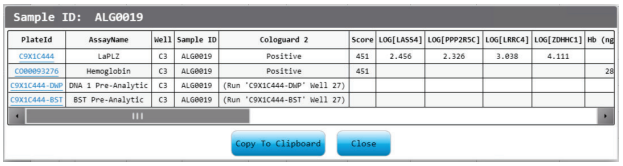


Figure 11-6: Sample ID Detail View

4. In the event of errors in manual processing steps or other errors observed, a Supervisor-level user may invalidate overall test results before they are dispositioned, except when configured for automatic overall result disposition.
   a. To invalidate an overall test result for a sample, go to the Overall Results view, select the checkbox next to the sample, and select the 'Invalidate…' button. A confirmation screen appears. Select 'Yes' to confirm and enter your comment, username, and password to complete the action.
   NOTE: *Samples invalidated by a user are permanently marked as invalid once a Disposition is performed and sample results are released.*
   b. To undo user invalidation, select the checkbox for the individual sample and select the 'Undo Invalidate…' button and enter your username and password to complete the action.
5. To disposition the overall results for a sample or group of samples, select the checkbox(es) beside the sample(s) and select the 'Disposition…' button on the bottom of the screen.
   NOTE: *The checkbox column header can be selected to mark all the samples displayed on the current page of the Overall results table (up to 100 per page) in order to release or close multiple results simultaneously.*

   NOTE: *If all selected results cannot be released, the 'Release' button will not be available.*

   Enter Supervisor-level username and password, then:
   a. Select 'Release' to release overall test results for export. Released overall test results are automatically written to LIS Export file.
   b. Alternatively, select 'Close' to indicate that the results should not be exported. Closed results cannot be exported in LIS Export.

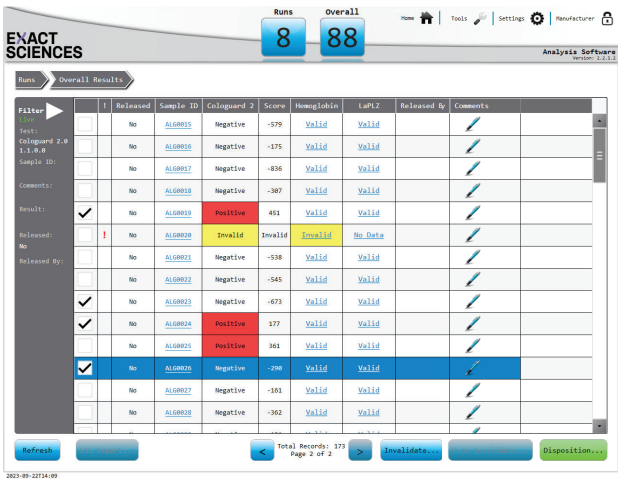


Figure 11-7: Dispositioning Overall Results for a Sample or a Group of Samples

12 Interpretation of Results

The Exact Sciences System Software imports run data into the Analysis Software. The software calculates assay results for controls and individual samples. Results of each assay, molecular and hemoglobin, are reviewed prior to the release of the assay run data in the software. Prior to releasing assay run results, deviations to process in the assay set up are noted for affected samples. Any deviation (e.g., operator error, instrument error) that occurs, may compromise the results of the test regardless of control validity. If a sample result is known by the user to be compromised, the user may invalidate the individual result. Results calculated as invalid by the system are automatically invalidated. Results may be released for the entire assay run. If an assay control fails, or the operator invalidates an entire assay run, no sample results will be present for that assay, and all samples in the run must be retested in order to generate an overall result. Only valid sample results from valid assay runs are used to calculate an overall Cologuard Plus score.

Users may also review overall Cologuard Plus test results and invalidate sample results as needed based on events and issues known to the user, such as individual sample or reagent contamination, process errors, or automation abort with unconfirmed completed steps. Users may enter comments in the software for any sample result that is invalidated by the user for a known technical error that cannot be detected by the software. An invalid overall result is generated for each sample where any assay of the sample in the plate run is invalid. Valid overall results wait as pending in the matching queue until a complete set of valid assay results are available for the sample, then the overall test result for the sample is calculated.

As users release valid molecular and hemoglobin results for an assay run, the Analysis Software will link the constituent assay results by sample ID and calculate a Cologuard Plus score and assign the final Cologuard Plus test result: Positive, Negative, or Invalid.

13 Procedural Notes and Precautions

13.1 Additional Stool Homogenate Aliquots

If additional aliquots of stool homogenate are required for testing or for sample archive, label and prepare additional tubes as in Preparation of Stool Homogenate for DNA Testing and store as directed.

NOTE: *Do not store samples in a Stool Container. Any additional homogenate aliquots should be processed and stored in 50 mL tubes at the same time as testing aliquots.*

13.2 Enter Supplemental Lot Information

Use the following procedures to support STARlet Setup when required.

1. Log in to the Analysis Client Software.
2. Import a .slib file into the Analysis Client Software.
3. Supplemental lot information loaded into the system can be viewed in the SLIB summary table by selecting the ‘SLIB’ button in the Analysis Software.

14 Quality Control

14.1 Calibration Acceptance Criteria

The assays in the Cologuard Plus test calculate marker result values using calibration curves created for each assay run. To ensure valid results, each calibration curve must meet specific acceptance criteria. These criteria are pre-defined in the Cologuard 2 Test Definition and may be useful in troubleshooting situations.

For the Molecular Assay, a linear least squares regression is calculated for each marker from calibrator values to calculate sample concentrations from the slope and intercept of each calibration curve. The calibration curves are generated by three levels of calibrators run in duplicate. Before fitting the linear regression, calibrator values are assessed for outliers. To account for the covariance of the slope and intercept, a tolerance ellipse approach is used to assess the model. For each marker, a valid calibration curve must meet the following criteria:

- A minimum of 5 calibrator replicates must be used in the model.

- Slope and intercept must fall within the pre-defined tolerance ellipse.

For the Hemoglobin Bead Based Assay, a four parameter curve fit with A=0 (see equation below) is calculated from calibrator values to calculate sample concentrations against the calibration curve.

$$Y = \frac{A - D}{1 + \left(\frac{X}{C}\right)^{B}} + D$$

Equation 1: Four parameter curve fit used to calculate Hemoglobin concentration, where X is concentration, Y is absorbance and A, B, C, and D are curve fit coefficients.

A weighted R-squared value is calculated to ensure that the curve fit is close to the measured calibrator values and fits through all seven calibration points. In addition, a minimum absorbance value must be achieved to ensure sufficient signal to noise ratio.

14.1.1 Process Controls

1. Process controls must be present in each assay plate setup run to achieve valid results. The system software will not proceed with the method if all required controls are not present.
   a. Input samples for Captured Sample Transfer must include CG2 D CTRL 1, CG2 D CTRL 2, CG2 D CTRL 3, and CG2 D CTRL 4 in each molecular run setup.
   b. Hemoglobin Controls (CG2 CTRL 1, CG2 CTRL 2, and CG2 CTRL 3) are required for each hemoglobin run setup.
2. Process controls must yield expected results, or the assay run will be invalid. Allowed ranges for control results are defined by the CG2 DNA Controls Supplemental Lot Information and CG2 Hemoglobin Bead Controls Supplemental Lot Information for each lot of controls.

15 Limitations of the Procedure

- The barcoded identification sequence scanned by the STARlet for hemoglobin sample and the DNA sample from a single Collection Kit must match for results to be matched into an overall Cologuard Plus test result.
- Automated procedures reduce the risk of contamination during the laboratory procedure. However, good laboratory practice and careful adherence to procedures in this document are important to reduce further risk of nucleic acid and protein contamination from calibrators, positive controls, or specimens.
- Invalid results could occur from improper handling or storage, technical error, or sample or control mix up. Ensure that only trained personnel perform the laboratory procedure.
- The Cologuard Plus test results are qualitative. The numeric value of the Cologuard Plus Score is not indicative of extent of disease.
- A negative test result does not exclude the possibility that the patient may have a precancerous or cancerous lesion. A false negative result with the Cologuard Plus test could potentially delay colonoscopy and a potentially delayed diagnosis of disease.
- A positive Cologuard Plus test suggests the presence of precancerous lesions and/or cancer. A false positive result could result in an additional invasive screening procedure for the patient, such as colonoscopy, and thus expose patients to the attendant risks associated with such a procedure.

16 Performance Characteristics

A summary of the findings from the analytical and clinical performance studies of the Cologuard Plus test is described below.

16.1 Algorithm Development and Clinical Cutoff Determination

A study was conducted in order to establish an algorithm and clinical decision point (cutoff value) for the Cologuard Plus test. The study included 3,011 samples: 100 CRC, 242 APL, 813 non-advanced precancerous lesions, and 1,856 negatives. Models were fit via nominal logistic regression, general additive model, neural net, and random forest. The logistic regression model was selected as it provided the best clinical performance with the least complexity. The cross-validated results for this final algorithm were 91.9% CRC sensitivity, 40.7% APL sensitivity, 90.9% specificity for negatives alone, and 88.5% for negatives and non-APL; these values aligned with point estimates.

16.2 Analytical Sensitivity

16.2.1 Molecular Assay Analytical Sensitivity and Linearity

The Limit of Detection (LoD), Limit of Quantitation (LoQ), linearity, and linear range were determined for the Molecular Assay component of the Cologuard Plus test. A summary of the results of this study is in Table 16-1.

The study included a minimum of six days, two reagent lots, one instrument, three pooled patient samples, and six dilutions per sample. Samples were prepared using sDNA from patient samples from the Cologuard Plus process. Blank samples had no detected signal in the LQAS assay. Therefore, the LoD and LoQ values were established through means independent of the LoB measurement, defined as the concentration of DNA where 95% of runs are detected at or below that concentration. To show that blank samples result in limited signal, the data for all no-template controls included on the 18 LoD/LoQ plates (n=72) were evaluated. An LoB of 0 strands was confirmed for all 4 markers.

The LoD is the concentration corresponding to 95% detection probability. The concentration where the robust CV falls below 20% is the LoQ. Established LoD and LoQ values are listed in the table below.

The linearity and linear range study was conducted using two lots of reagents, one QS5Dx instrument and one operator. Two PCR plates were setup per reagent lot for a total of four plates. Two dilution series were prepared using two unique sample pools. The sample pools were prepared from spiked and unspiked endogenous pools diluted with blank diluent.

The linear range was determined as the lowest or highest point that provided results within the pre-specified allowable deviation from linearity (ADL). If any of those values fell below the LoD of the algorithm, the lower algorithm cutoff was claimed. The established linear range for each marker is outlined in the table below.

Table 16-1: Molecular Assay Analytical Sensitivity Characteristics Summary

Performance Characteristic	Result
Limit of Detection	LoD determined at level where 95% detection was met. 3 strands for LASS4 2 strands for PPP2R5C 2 strands for LRRC4 2 strands for ZDHHC1
Limit of Quantitation	<20% CV at LoQ concentrations 25 strands for LASS4 21 strands for PPP2R5C 27 strands for LRRC4 14 strands for ZDHHC1
Linear Range	9–1,380,384 strands for LASS4 5–1,318,257 strands for PPP2R5C 5–1,380,384 strands for LRRC4 250–100,000 strands for ZDHHC1

Based on the study data, the LoD claims of the Molecular Assay for LASS4, PPP2R5C, and LRRC4 are ≤5 strands and the LoD claim for ZDHHC1 is ≤251 strands. For the reference marker ZDHHC1, samples with strand values < 2.4 log strands (approximately 251 strands) are called invalid.

16.2.2 Hemoglobin Assay Sensitivity and Linearity

The LoB, LoD, LoQ, linearity, linear range, and Hook Effect were determined for the Hemoglobin (Hb) assay component of the Cologuard Plus test. A summary of the results is in the Table 16-2 below.

The LoB, LoD, and LoQ study was conducted using two lots of reagents on a single instrument for four runs per reagent lot. Samples for the LoD and LoQ study were made from pools of stool samples with endogenous Hb and diluted to near LoD/LoQ levels using unique lots of Reconstitution Buffer. The 95th percentile of 80 replicates of Reconstitution Buffer for blank measurements was determined to be the LoB. LoD and LoQ were established with 64 replicates of four unique patient samples for each of the four concentration levels of 10.0, 7.5, 5.0, and 2.5 ng/mL. The concentration of Hb at which at least 95% of runs were above the LoB was determined as the LoD. LoQ was determined to be the concentration of Hb at which the CV is below 20%, is greater than or equal to LoB, and where LoD is not larger than LoQ.

The Hook Effect was assessed by testing four replicates of each of the 10 Hb concentration levels above, below, and spanning the anticipated quantitative range of one normal blood sample (HbA) and two hemoglobin variants (HbS and HbC). The mean values for all samples with concentrations above the upper algorithm limit of 1,000 ng/mL were compared for a decrease in signal. There was no bias from Hook effect for Hb concentration of up to 100,000 ng/mL which included the 1 mg per gram of stool (10 µg Hb input into assay) pre-specified in the acceptance criteria.

For the linearity study, two unique samples were diluted to 9 Hb concentration levels spanning the anticipated quantitative range of 10-1,000 ng/mL. A minimum of 4 replicates were tested for each sample and level. A

linear regression function was fit for each sample. For each dilution level, the predicted value was compared to the mean of the repeats of that dilution. The difference was compared to a pre-specified allowable deviation from linearity (ADL). The anticipated quantitative range of 10–1,000 ng/mL was identified to be within the ADL.

Table 16-2: Hemoglobin Assay Analytical Sensitivity Characteristics Summary

Performance Characteristic	Analytical Sensitivity Study Result
Limit of Blank	2.0 ng/mL
Limit of Detection	2.9 ng/mL
Limit of Quantitation	2.9 ng/mL
Linearity	Linear range = 10–1,000 ng/mL
Hook Effect	No Hook Effect observed

16.3 Interfering Substances

This study evaluated the impact to the Cologuard Plus Score due to interfering substances found in stool through ingestion or external application. Substances included common medications (such as antacids, antibiotics, anti-inflammatories, anti-fungal medications, pain relievers, decongestants, stool softeners, anti-diarrheal medications, and laxatives), urine, ethanol, cholesterol and fatty acids, vitamin C, iron, a mixture of fruits and vegetables, genomic DNA from common edible animals, hypomethylation agents, and DNA stabilization buffer in the Hb assay. High negative and low positive stool pools were prepared with and without the presence of these substances, and 10 replicates of each sample were tested in the molecular and hemoglobin assays. No meaningful amount of interference was detected for any interfering substances.

16.4 Specificity and Cross-Reactivity

This testing included an assessment of cross-reactivity of cancers and diseases other than colorectal cancer and analytical specificity of the methylation and hemoglobin markers that are detected by the Cologuard Plus test.

16.4.1 Non-Colorectal Cancers and Diseases

Specificity of the Cologuard Plus test was evaluated using sample specimens collected from subjects with 12 cancer and disease groups other than colorectal cancer (CRC). The table below indicates the final number of cancer or disease patient samples that were tested.

The false positive fraction (FPF) of test results was calculated as a point estimate and a two-sided 95% confidence interval for each disease group. Each FPF was compared to the estimated FPF for the general intended use (IU) population. The disease groups of lung cancer, esophageal cancer, and inflammatory bowel disease did not overlap the estimated FPF for the general IU population. The other nine groups had observed positive test results rates that are consistent with the FPF for the overall assay.

For the assay specificity analysis, the total number of positive calls per 10,000 patients was estimated to be 8.1 to 9.0 with the inclusion of IBD and 7.7 to 8.0 without, as shown in the following table. This was considered a negligible effect on the Cologuard Plus test positivity.

Table 16-3: Cancers and Diseases Tested for Cross-Reactivity

No.	Cancer or Disease[a]	No. of Valid Samples Tested	Incidence per 10,000 population[b]	% Positivity of Cologuard Plus Result	No. Positive Cologuard Plus Calls in 10,000 Patients
1	Autoimmune Disease[c] (individual disease not specified)	29	3.2–5.4	13.8	0.4–0.7
2	Bladder Cancer	5	1.8	20.0	0.4
3	Breast Cancer	35	12.6	11.4	1.4
4	Esophageal Cancer	11	0.4	36.4	0.1
5	Gynecologic Cancer (i.e., endometrial cancer, vulvar melanoma, and ovarian cancer	41	3.8	4.9	0.2
6	Hepatic Cancer (i.e., liver and bile duct cancer)	5	0.9	20.0	0.2
7	Inflammatory Bowel Disease[c]	30	1.5–3.9	26.7	0.4–1.0
8	Kidney/Renal Pelvis Cancer	20	1.7	10.0	0.2
9	Lung Cancer	30	5.0	33.3	1.7
10	Pancreatic Cancer	13	1.3	15.4	0.2
11	Prostate Cancer	35	11.3	22.9	2.6
12	Stomach Cancer	5	0.7	40.0	0.3
Total (with IBD)					8.1–9.0
Total (without IBD)					7.7–8.0

a USA population-based cancer incidence data were obtained from registries that participate in the CDC's National Program of Cancer Registries and/or the NCI's Surveillance, Epidemiology, and End Results (SEER) Program.

b Cancer prevalence or incidence per 10,000 population was calculated with the assumption the population consists of 50/50 male-to-female.

c Incidence of autoimmune diseases reported for North America include Multiple Sclerosis, Type I Diabetes, Primary Biliary Cirrhosis, Autoimmune Hepatitis, Graves' Disease, Coeliac Disease, Addison's Disease, Sjogren's Syndrome, Systemic Lupus Erythematosus and Rheumatoid Arthritis. See Wang, L., Wang, F., and Gershwin, M.E. (2015). Human autoimmune diseases: a comprehensive update. Journal of Internal Medicine, Volume 278, Issue 4, Pages 369-395.7

16.4.2 Analytical Specificity

The Cologuard Plus Hemoglobin Assay is designed to detect patient-origin Hb in human stool and the Molecular Assay is designed to detect only fully methylated LASS4, PPP2R5C, LRRC4 and ZDHHC1 gene targets.

The Hemoglobin Assay was tested for cross-reactivity with Hb and Myoglobin (Mb) from animals that could be present in a human stool sample due to diet, and the Molecular Assay was tested with fully unmethylated DNA target sequences that are likely to be present as background in all patient samples. The Hemoglobin Assay had 10 replicates of each sample both unspiked and spiked with whole blood, Mb from meat extracts, or purified Mb from eight commonly eaten animal species (bovine, pig, turkey, chicken, trout, goat, rabbit, and sheep). The Molecular Assay had 45 replicates of each sample, both unspiked and spiked with synthetic, fully unmethylated DNA target sequences of each of the methylation markers combined into a single sample type.

Both marker-level and score-level assessments showed minimal cross-reactivity below the specified acceptance criteria to non-human Hb, non-human Mb for the Hemoglobin Assay and to unmethylated target sequences for the Molecular Assay.

16.5 Precision

16.5.1 Precision and Reproducibility Study with Clinical Samples

This study examined reproducibility between three laboratory sites using a panel of clinical samples. At each site, two operators performed testing for five non-consecutive days for a total of five assay runs. The sample panel consisted of six clinical samples prepared from de-identified patient specimens, and one fully synthetic control sample. The panel represented a range of pathologies including CRCs, APLs, and negatives with varying levels of marker signals and Cologuard Plus scores representing a wide range of test results including samples close to the algorithm cutoff (see Table 16-4).

Table 16-4: Reproducibility and Precision (Sample Panel Overview)

Sample Type	Pathology Type	Sample Matrix	Expected Result	Replicates per Run	Replicates per Site	Replicates Across Sites
High CRC Stool	CRC Stage III	Stool	Positive	6	30	90
Low CRC Stool	CRC Stage I	Stool	Positive	6	30	90
High APL Stool	Advanced Adenoma	Stool	Positive	6	30	90
Low APL Stool (C95)	Advanced Adenoma	Stool	Positive	6	30	90
High Negative Stool (C5)	Non-advanced Adenoma	Stool	Negative	6	30	90
Low Negative Stool	Negative	Stool	Negative	6	30	90
Low Positive Control	NA	Control	Positive	6	30	90

Percent agreement values were calculated between observed and expected Cologuard Plus test results, yielding 100% agreement for positive samples, 96.6% agreement for negative samples, and 99.0% agreement for the pooled results. The lower 95% confidence limit for total percent agreement was greater than 95% for all samples. Precision also exceeded 95% for all laboratory sites (see Table 16-5). In the design for this study, site was confounded with operator and instrument, and run was confounded with day.

Table 16-5: Percent Agreement by Site

	Percent Agreement (%)	Lower 95% CI (%)
Overall	99.0	98.1
Positive	100.0	99.3
Negative	96.6	93.5
Site 1	99.0	97.0
Site 2	98.1	95.7
Site 3	100.0	98.6

Four sample types — the High Negative Stool (C5), Low APL Stool (C95), High APL Stool , and Low Positive Control had mean Hb concentrations less than 300 ng/mL and mean AvgMDM values greater than 0 and thus were subject to the acceptance criterion. These samples showed a maximum upper 95% CI SD of 39 (see Table 16-6).

Table 16-6: SD of Cologuard Plus Score and Upper 95% CI of SD

Sample	N	Mean	SD	Upper 95% CI
High CRC Stool	89	2031	64	73
Low CRC Stool	89	1169	42	48
High APL Stool	90	371	20	23
Low APL Stool (C95)	89	102	22	26
High Negative Stool (C5)	90	-56	34	39
Low Negative Stool	89	-382	76	86
Low Positive Control	90	214	23	26

16.5.2 Precision and Reproducibility Study with Contrived Samples

This study examined reproducibility between 3 laboratory sites, a minimum of 2 operator groups per site, and 2 instrument groups per site. Testing was performed across 22 assay runs at each site using five samples consisting of pooled stool with synthetic DNA spikes, and three synthetic controls prepared with varying levels of marker signals and Cologuard Plus scores representing a wide range of test results including samples close to the algorithm cutoff (see Table 16-7).

Table 16-7: Precision and Reproducibility Sample Panel Overview

Sample Type	Pathology Type	DNA Sample Matrix	Hb Sample Matrix	Expected Result	Replicates per Run	Replicates per Site	Replicates Across Sites
High positive stool	CRC Stage III	Stool	Stool	Positive	6	132	396
Mid positive stool	CRC Stage I	Stool	Stool	Positive	6	132	396
Negative stool	Negative	Stool	Stool	Negative	6	132	396
C5[a]	Non-advanced Adenoma	Stool	Stool	Negative	6	132	396
C95[b]	Advanced Adenoma	Stool	Stool	Positive	6	132	396
High Positive Control	NA	Buffer	Buffer	Positive	5	110	330
Low Positive Control	NA	Buffer	Buffer	Positive	5	110	330
Negative Control	NA	Buffer	Buffer	Negative	4	88	264

a 5% of pool replicates are expected to have a positive test result due to measurement error.

b 95% of pool replicates are expected to have a positive test result and 5% are expected to have a negative result due to measurement error.

Percent agreement values were calculated between observed and expected Cologuard Plus test results, yielding 100% agreement for positive samples, 98.9% agreement for negative samples, and 99.6% agreement for the pooled results. The lower 95% confidence limit for call concordance exceeded 95% for all samples. Precision also exceeded 95% for all operators, instruments, and laboratory sites. (see Table 16-8).

Table 16-8: Call Concordance Between Sites, Operators, and Instruments

Comparison	Pairs of samples	Sample pairs match	Agreement	Lower 95% CI
Site 2 vs Site 3	959	950	99.1%	98.2%
Site 2 vs Site 1	949	941	99.2%	98.3%
Site 3 vs Site 1	949	946	99.7%	99.1%
Site 1 Operators	466	463	99.4%	98.1%
Site 2 Operators	475	467	98.3%	96.7%
Site 3 Operators	475	474	99.8%	98.8%
Site 1 Instruments	466	463	99.4%	98.1%
Site 2 Instruments	475	467	98.3%	96.7%
Site 3 Instruments	475	474	99.8%	98.8%

Four sample types with mean Hb concentrations less than 300 ng/mL and mean AvgMDM (median weighted average of the reference-normalized, standardized methylation marker DNA concentrations) values greater than 0 (C5, C95, High Positive Control, and Low Positive Control) were subject to the SD acceptance criterion. These samples showed a maximum upper 95% CI SD of 39 (see Table 16-9).

Table 16-9: SD of Cologuard Plus Score and Upper 95% CI of SD

Sample	N	Mean Score	SD Score	Upper 95% CI
High Positive Control	328	1113	24	26
C5	390	-69	36	39
C95	394	94	24	26
Low Positive Control	329	225	22	23
High Positive Stool	392	2050	81	87
Mid Positive Stool	395	1369	49	53
Negative Control	263	-430	23	25
Negative Stool	394	-469	52	55

The lot-to-lot reproducibility of the molecular and hemoglobin (Hb) assay reagents was assessed to demonstrate that the 95% lower confidence limit on the percent agreement between reagent lots was ≥95%. A single site study was performed with three reagent lots made with unique raw materials where possible and three lots of consumables. Three runs per reagent lot were completed for each assay.

The sample panel used in this study included five contrived samples and three control samples, possessing varying levels of methylated DNA markers (MDMs) and Hb concentration to provide a range of Cologuard Plus scores. The targeted scores were chosen to mimic clinical specimens representative of the intended use population and to encompass samples with scores near the algorithm cut-off. The Table 16-10 below outlines the number of replicates analyzed for each sample type included in this study.

Table 16-10: Summary of Lot-to-lot Reproducibility Using Contrived Samples

Sample Type	Pathology type	DNA sample matrix	Hb Sample Matrix	Expected Result	Replicates per Run	Replicates per Reagent Lot	Replicates Across Lots
High positive stool	CRC Stage III	Stool	Stool	Positive	6	18	54
Mid positive stool	CRC Stage I	Stool	Stool	Positive	6	18	54
Negative stool	Negative	Stool	Stool	Negative	6	18	54
C5	Non-advanced Adenoma	Stool	Stool	Negative	6	18	54
C95	Advanced Adenoma	Stool	Stool	Positive	6	18	54
High Positive Control	NA	Buffer	Buffer	Positive	5	15	45
Low Positive Control	NA	Buffer	Buffer	Positive	5	15	45
Negative Control	NA	Buffer	Buffer	Negative	4	12	36

Four samples had Hb <300 ng/mL and AvgMDM >0, and thus were subject to the SD acceptance criterion. All results passed, as outlined in the Table 16-11.

Table 16-11: SDs of Sample Types

Sample Type	N	Mean Score	SD Score	Upper 95% CI
C5	53	-83	38	47
C95	54	94	23	28
High Positive Control	44	1091	26	33
High Positive Stool	54	2083	57	70
Low Positive Control	45	218	23	29
Mid Positive Stool	52	1393	53	66
Negative Control	36	-429	29	38
Negative Stool	54	-492	58	72

Additionally, all samples were found to have ≥95% concordance with the expected calls per sample type as shown in Table 16-12 below.

Table 16-12: Concordance Values

Condition	Concordance	Lower 95% CI	N
Total	100%	99.1%	392
Lot 1 vs Lot 2	100%	98.6%	262
Lot 1 vs Lot 3	100%	98.6%	260

Condition	Concordance	Lower 95% CI	N
Lot 2 vs Lot 3	100%	98.6%	260

16.5.3 Specimen Reproducibility

This study examined assay performance and call concordance with stool samples of known pathology (10 CRC, 10 APL, and 10 Negative) for 30 individual subjects. For each individual subject, three stool homogenates aliquots from the same whole stool collection kit and three aliquots of fecal occult hemoglobin from the same FIT tube were tested through the Cologuard Plus workflow.

Samples were selected to represent a range of disease states, a range of molecular marker and Hb values, and a range of Cologuard Plus scores, including some near the assay cut-off.

Call concordance between the three aliquots was 100% for all but two samples, both of which were normal (negative) samples near the clinical decision point.

Table 16-13: Sample Panel Results

Subject ID	Pathology	Category	Stage	Mean Score	Standard Deviation Score	CV Score	N Valid	N Pos	N Neg	% Concordant
150HTWD	CRC	1	Stage I	1502	15	1	3	3	0	100%
150HZO8	CRC	1	Stage I	83	58	70	3	3	0	100%
150R0D7	CRC	1	Stage I	308	19	6	3	3	0	100%
150R0K5	CRC	1	Stage I	807	18	2	3	3	0	100%
160AVST	CRC	1	Stage I	935	15	2	3	3	0	100%
160CIOK	CRC	1	Stage I	1394	33	2	3	3	0	100%
170C3T1	CRC	1	Stage I	1180	26	2	3	3	0	100%
150GBF1	CRC	1	Stage II	1755	10	1	3	3	0	100%
150SBSC	CRC	1	Stage II	1221	2	0	3	3	0	100%
150KH7K	CRC	1	Stage III	1096	23	2	3	3	0	100%
150YAOD	APL	2.1	N/A	286	43	15	3	3	0	100%
150HL8Q	APL	2.2	N/A	475	14	3	3	3	0	100%
150VG3M	APL	2.2	N/A	779	14	2	3	3	0	100%
150XXKZ	APL	2.2	N/A	254	22	9	3	3	0	100%
1602UA0	APL	2.2	N/A	335	6	2	3	3	0	100%
150FETS	APL	2.3	N/A	95	18	19	3	3	0	100%
150LF27	APL	2.3	N/A	1532	70	5	3	3	0	100%
150S16G	APL	2.3	N/A	986	36	4	3	3	0	100%
150ZWCA	APL	2.3	N/A	343	21	6	3	3	0	100%
150NCZB	APL	2.4	N/A	184	5	3	3	3	0	100%
150S18F	Normal	3	N/A	-86	78	-90	3	0	3	100%
150IG05	Normal	4	N/A	-26	102	N/A[a]	3	1	2	67%
16028XZ	Normal	5	N/A	-312	36	-12	3	0	3	100%
150IFZ9	Normal	6.1	N/A	-158	50	-32	3	0	3	100%

Subject ID	Pathology	Category	Stage	Mean Score	Standard Deviation Score	CV Score	N Valid	N Pos	N Neg	% Concordant
150L83S	Normal	6.1	N/A	6	21	N/A[a]	3	2	1	33%
15011VM	Normal	6.1	N/A	-68	32	-47	3	0	3	100%
150JZBF	Normal	6.2	N/A	-666	175	-26	3	0	3	100%
150KTLZ	Normal	6.2	N/A	-245	40	-16	3	0	3	100%
150UCH2	Normal	6.2	N/A	-388	54	-14	3	0	3	100%
1504A3Y	Normal	6.2	N/A	-464	34	-7	3	0	3	100%

[a] CVs not calculated as score replicates span zero.

16.6 Robustness

This study evaluated the robustness of the Cologuard Plus test in response to variation in specific steps in the molecular and hemoglobin assay procedures. Specifically, several steps in the Cologuard Plus workflow require user handling, such as sample handling, reagent aspiration, and reagent dispensing, and variability in these steps could affect the test result. Testing was performed using three operators for the molecular testing and two operators for the hemoglobin testing. One set of instrumentation was used for each test factor, and single reagent lot was used for the study.

Factors tested in the Molecular Assay included the following:

- Variations in volume adjustment of clarified supernatant and amount of addition of Capture Beads and Capture Wash.
- Time delay in addition of Capture Beads, Capture Solution, and Capture Wash.
- Time delay to start capture incubation after addition of Capture Beads and Capture Solution while on bench and while in incubator.
- Time delay to load LQAS reagents onto Hamilton STARlet, and to load unsealed LQAS plate into QS5 Dx.

Factors tested in the Hemoglobin Assay included the following:

- Variation in volume of Reconstitution Buffer addition to calibrators and controls.
- Time delay to read plate on plate reader.

The results of this study assessing variation at specific steps requiring user handling showed that for all the robustness factors, the Cologuard Plus scores for the test condition were within the pre-specified acceptance criteria (the mean Cologuard Plus score for each sample was +/- 80 units from the mean Cologuard Plus score for the standard condition).

16.7 Carry-over and Cross-contamination

This study examined the impact of carry-over and cross-contamination of the Cologuard Plus workflow on assay results. Testing was initiated with a single assay run with negative samples (control stage), followed by five assay runs comprised of alternating negative and high positive samples prepared in a checkerboard sequence (test stage). Assay results were used to calculate a Cologuard Plus score and qualitative test result. Positive test rates of the negative samples were compared between the test and control stage to test for non-inferiority as a measure of clinical significance due to contamination. Results showed no difference in the positive test rate between the test and control stages, with no incorrect calls. Cross-contamination was also examined per marker, except for reference marker ZDHHC1, by comparing the difference in mean marker signal observed in the control stage and test stage for the high negative samples. The upper bound of the difference between the test and control stages was less than 1% of the high positive mean. Results from the analysis demonstrated an acceptably low level of carry-over and cross-contamination in the Cologuard Plus workflow.

16.8 Stability Studies

16.8.1 Sample Stability

Testing was performed to establish the in-process specimen stability at various stopping points in the Cologuard Plus workflow. These included:

- Stability of DNA hybridized to capture probes conjugated to magnetic beads (captured DNA) at room temperature (0, 3, 6, 8, and 9 hours)
- Stability of eluted, bisulfite-converted DNA at 2-8°C (1, 2, 3, 4, and 5 days)
- Stability of PVPP treated (clarified) stool supernatant at room temperature (0, 2, 4, 6, and 7 hours)

- Stability of thawed stool homogenate at room temperature (0, 2, 4, 6 and 7 hours)
- Stability of the Hb tube at 2-8°C (0, 3, 7, 10, 14, and 15 days)
- Stability of the Hb tube at room temperature (0, 6, 12, 18, 24 and 25 hours)

Samples for the Molecular Assay included a negative synthetic control and a positive pooled clinical sample. For the Hemoglobin Assay, samples included pooled clinical samples with low and high Hb levels. Greater than or equal to 12 replicates were tested at each time point, and stability was evaluated by using linear regression to model the effect of time on the Cologuard Plus score and / or marker concentrations.

The results of the studies demonstrated all conditions met the pre-specified acceptance criteria at all time points, supporting a claim of the penultimate time point for each condition tested.

16.8.2 Reagent Stability

In-Use Reagent Stability

In-use reagent stability testing was performed to establish the stability recommendations for multiple-use reagents and controls once reagent containers had been opened and for on-deck automation reagents once they were poured into troughs or placed on-deck prior to run initiation. Testing was performed separately for the molecular and hemoglobin portions of the Cologuard Plus test.

The multiple-use reagents and controls were tested at 7 time points (0, 31, 41, 62, 72, 93, and 100 days). The on-deck automation reagents were tested at 5 time points (0, 2, 4, 6, and 7 hours). For all reagents, a total of 13 replicates per sample type were run at each time point. To determine stability, linear regression was used to model the effect of time on the Cologuard Plus scores or marker concentrations.

The results of the study demonstrated that all reagent groups met the pre-specified acceptance criteria for stability at all time points , supporting a claim of 3 months in-use stability for the multiple-use reagents and controls, and 6 hours for the on-deck automation reagents.

Real-Time Reagent Stability

A real-time stability study is being run to establish the stability of the Cologuard Plus test Molecular Assay reagents and DNA controls, as well as the Cologuard Plus test /Hemoglobin Assay reagents and controls. The study plans to evaluate the functional performance of three reagent lots over the course of 27 months, with the goal of establishing a minimum 24-month stability of the on-test reagents and controls. The following stability metrics will be measured: Cologuard Plus score, AvgMDM and *ZDHHC1* log strands for the Molecular Assay Reagents; Cologuard Plus Score and hemoglobin concentration for the Hemoglobin Assay Reagents; log strands of the DNA markers for the Molecular Assay DNA Controls; and hemoglobin concentration for the Hemoglobin Assay Controls. Interim analyses support a stability duration of at least six months for all reagents and controls.

Freeze/Thaw Reagent Stability

A freeze/thaw stability study was performed to evaluate the stability of LQAS reagents stored at -20°C. Four conditions were evaluated in the study: 0, 2, 4, and 6 freeze/thaw cycles. Additionally, two sample types were tested: synthetic target in a run control matrix, intended to provide a negative Cologuard Plus score, and endogenous target in a stool matrix, intended to provide a positive Cologuard Plus score. To determine stability, linear regression was used to model the effect of the number of freeze/thaw cycles on the Cologuard Plus score.

All reagent groups met the pre-specified acceptance criteria for stability at all time points, demonstrating that the LQAS reagents are stable for up to four freeze/thaw cycles.

16.8.3 Shipping Stability

Testing was performed to evaluate the stability of the Hb and whole stool samples under shipping stress conditions. The Hemoglobin and Molecular Assay samples were subjected to ship stress conditions, and evaluated for a period of 0, 3, 4, 5, 6, and 7 days, and 0, 1, 3, 5, 8, and 9 days, respectively. The Hb study used pooled clinical samples with low, mid, and high Hb levels, and evaluated 16 replicates at each time point. The DNA study used a panel of 20 positive and negative clinical samples and evaluated 3 replicates at each time point. Stability was assessed by using linear regression to model the effect of time on the Cologuard Plus score and / or marker concentrations.

The results of the studies demonstrated all conditions met the pre-specified acceptance criteria at all time points, supporting a claim of 6 days for the Hb sample, and 8 days for the DNA sample.

16.9 Clinical Sensitivity and Specificity

The clinical performance of the Cologuard Plus test was evaluated in the prospective, cross-sectional, multi-center, pivotal study, named BLUE-C.[6]The study

compared the performance of the Cologuard Plus test with a commercially available independent Fecal Immunochemical Test (FIT) (Polymedco OC-Auto® Micro 80 iFOB test) for colorectal cancer (CRC) and advanced precancerous lesion (APL) detection, with colonoscopy as the reference method.

The primary objective was to assess the sensitivity for CRC detection and specificity of the Cologuard Plus test. The secondary objectives were to assess the sensitivity of the Cologuard Plus test for APL detection; compare the sensitivity for CRC and APL detection of the Cologuard Plus test to a commercially available FIT; and evaluate the specificity of the Cologuard Plus test for participants with no colorectal neoplastic findings.

16.9.1 Study Design

The BLUE-C study enrolled a total of 26,758 participants at 186 sites in the United States (ClinicalTrials.gov, Trial Registration ID: NCT04144738). Participants were considered enrolled if they met all eligibility criteria during screening and provided written informed consent. Participants were provided with a stool collection kit, which included collection materials for Cologuard Plus, a commercial FIT, and sample collection instructions to complete the stool collection prior to bowel preparation for the colonoscopy procedure. Histopathological information was collected for tissue removed during colonoscopy and, if applicable, any follow-up procedures. Colorectal lesions identified during colonoscopy were categorized based on the most clinically significant lesion present (Index Lesion), as indicated in Table 16-14. The American Joint Committee on Cancer (AJCC) Staging System, 8th edition, was used for recording CRC stages.[5]

Table 16-14: Participant Categorization Based on Histopathologic Diagnosis of the Index Lesion

Category	Description
1	Stage I-IV colorectal cancer, any size
2	Advanced Precancerous Lesions (APL), including the following subcategories:
2.1	High-grade dysplasia or ≥10 adenomas, any size
2.1a	High-grade dysplasia, any size
2.1b	≥10 adenomas, any size
2.2	Tubulovillous adenoma, any size
2.3	Tubular adenoma, ≥10 mm
2.4	Sessile serrated lesion with dysplasia (SSLD); Traditional serrated adenoma (TSA), Conventional adenoma with serrated architecture; Sessile serrated lesion; ≥10 mm
3	3-9 adenomas or sessile serrated lesions, <10 mm, non-advanced
4	1-2 adenomas or sessile serrated lesions, 5-9 mm, non-advanced
5	1-2 adenomas or sessile serrated lesions, <5 mm, non-advanced
6	Negative: no adenocarcinoma of the colorectum, no adenomas or SSA/SSP
6.1	Hyperplastic polyps or non-neoplastic lesions
6.2	No lesions on colonoscopy
X	Index Lesion could not be categorized because tissue/report was lost/not provided or histopathological diagnosis could not be determined.

Investigators and/or colonoscopists were blinded to all Cologuard Plus test and FIT results. Individuals conducting the Cologuard Plus test laboratory testing were blinded to all clinical data and to the results of the FIT. The Cologuard Plus test performance and FIT performance were assessed and compared to evaluate whether the study objectives were met.

16.9.2 Inclusion and Exclusion Criteria

Participants eligible for enrollment in the study were 40** years of age or older and were at average risk for development of CRC and asymptomatic for

** The enrolled patients who were between 40-44 years of age have been excluded from the data analysis.

gastrointestinal symptoms warranting diagnostic or therapeutic colonoscopy. Some of the exclusion criteria were participants who had a personal history of CRC or APL, a diagnosis of inflammatory bowel disease (IBD), a positive Cologuard test within the previous 3 years, or FIT within the previous 12 months.

16.9.3 Analyses Methods

The primary analysis population consisted of all enrolled participants with a valid Cologuard Plus test result, an evaluable colonoscopy, and meeting all study eligibility criteria. In addition to the study enrollment eligibility criteria, the primary analysis population excluded participants with a first-degree relative with CRC diagnosed at any age, as well as participants under the age of 45 years. The study was designed to have at least 85% power for all primary and secondary analyses

The two pre-specified primary endpoint hypotheses were (1) to test if the Cologuard Plus test sensitivity for CRC rejects the 75% null hypothesis, and (2) to test if the Cologuard Plus test specificity for participants without advanced neoplasia (CRC or APL) rejects the 85.9% null hypothesis. Each primary hypothesis was evaluated using a one-sided exact binomial test at the 2.5% significance level, corresponding to requiring the one-sided 97.5% exact binomial confidence bound (or, equivalently, the lower bound of the 2-sided 95% exact confidence interval (CI)) to be greater than the null hypothesis value. Both primary null hypotheses needed to be rejected for the study to be considered successful.

The four secondary endpoint hypotheses were (1) to test if the Cologuard Plus test sensitivity for participants with APL findings rejects the 38.9% null hypothesis, (2) to test if the Cologuard Plus test sensitivity for CRC detection is superior to that of a commercially available FIT, (3) to test if the Cologuard Plus test sensitivity for APL detection is superior to that of a commercially available FIT, and (4) to test if Cologuard Plus test specificity for participants with no colorectal neoplastic findings rejects an 87.5% null hypothesis.

Secondary hypotheses (1) and (4), were evaluated using a one-sided exact binomial test at 2.5% significance level, corresponding to requiring the one-sided 97.5% exact binomial confidence bound (or, equivalently, the lower bound of the 2-sided 95% exact CI) to be greater than the null hypothesis value.

The head-to-head comparisons with the commercial FIT were performed using exact McNemar's tests for paired proportions at the one-sided 2.5% significance level.

16.9.4 Participant Accountability

Of the total 26,758 participants enrolled in the study, 18,911 were included in the primary effectiveness population and 18,882 in the comparative effectiveness population. 5,573 participants were not included in the primary analysis population due not meeting analysis inclusion-exclusion criteria or not completing all study procedures. Of the remaining 21,185 participants who completed study procedures, 1,044 had exclusions related to stool sample or testing (139 stool samples collected after initial colonoscopy or bowel preparation, 807 unusable stool samples received, 98 invalid Cologuard Plus test results), and 1,230 were excluded for lack of an evaluable colonoscopy, resulting in 18,911 participants in the primary effectiveness population, of which 29 did not have a usable and valid FIT result, resulting in 18,882 participants in the comparative effectiveness population.

16.9.5 Demographic and Baseline Characteristics

The distribution of race and ethnicity among BLUE-C participants included in the primary effectiveness population closely mirrored that of the United States population, as reported in the 2020 Census results. The average age of participants was 63.0 years, and 53.1% of participants were female. The race and ethnicity distribution of participants was 59.7% White, not Hispanic or Latino; 16.4% Hispanic or Latino; 13.4% Black or African American, not Hispanic or Latino; and 9.0% Asian, not Hispanic or Latino. There was a small percentage of other race and ethnic participants including American Indian and Native Hawaiian participants included in the study. Average BMI was 29.5 kg/m^2 and 63.6% participants had never smoked. 32.0% of the participants had had a colonoscopy (>9 years prior to enrollment) in their lifetime and 3.8% had a prior Cologuard test.

16.9.6 Clinical Study Results

Data was analyzed for 18,911 participants meeting criteria for inclusion in the primary effectiveness population (see following table).

Table 16-15: Summary of the Cologuard Plus Test Performance.

Indication	Primary Effectiveness Population
Sensitivity %, (95% CI) (n detected/N)	
CRC	95.3 (88.4, 98.7) (81/85)
APL	43.3 (41.1, 45.5) (849/1,962)
Specificity % (95% CI) (n negative/N)	

Indication	Primary Effectiveness Population
Category 3–6	90.7 (90.3, 91.1) (15,297/16,864)
No colorectal neoplasia (Category 6)	92.7 (92.2, 93.2) (9,609/10,361)

The positive predictive value (PPV) of the Cologuard Plus test was 3.2% for CRC and 34.0% for APL. Among participants with a positive Cologuard Plus test result, 69.9% (1,745/2,497) were found to have a CRC, APL, or non-advanced adenoma. The negative predictive value (NPV) for CRC of the Cologuard Plus test was 99.98%, with only 0.02% of participants with a negative test result having CRC. (See table below).

Table 16-16: Positive and Negative Predictive Values: Index Lesion Categorization by Cologuard Plus Test Result

Index Lesion Categorization	Positive Predictive Value (PPV), % (95% CI); n/N positive test results	1-Negative Predictive Value (1-NPV), % (95% CI); n/N negative test results
CRC (n=85)	3.2 (2.6-4.0); 81/2,497	0.02 (0.01-0.06); 4/16,414
APL (n=1,962)	34.0 (32.1-35.9); 849/2,497	6.8 (6.4-7.2); 1,113/16,414
Category 3–5 (n=6,503)	32.6 (30.8-34.5); 815/2,497	34.7 (33.9-35.4); 5,688/16,414
Category 6 (n=10,361)	30.1 (28.3-32.0); 752/2,497	58.5 (57.8-59.3); 9,609/16,414

In the comparative effectiveness population, sensitivity for CRC was greater for the Cologuard Plus test compared to independent FIT (95.3% vs. 70.6%, respectively, exact McNemar p<0.0001). The Cologuard Plus test identified 21 of 25 (84.0%) CRC cases that were missed by FIT, while FIT did not identify any cancer cases that were not identified by Cologuard Plus. Sensitivity for APL was greater for the Cologuard Plus test compared to independent FIT (43.3% vs. 23.3%, respectively, exact McNemar p<0.0001). The Cologuard Plus test identified 506 of 1,503 (33.7%) APL cases missed by FIT, while FIT identified 115 of 1,112 (10.3%) APL cases missed by Cologuard Plus.

CRC and APL sensitivity was consistently higher for the Cologuard Plus test compared to independent FIT across cancer stages, lesion sizes, lesion locations, and APL subtypes as shown in following tables.

Table 16-17: The Cologuard Plus Test CRC Sensitivity by Colonoscopy Categories Compared to Independent FIT CRC Sensitivity with Confidence Intervals

CRC Subgroup	Cologuard Plus CRC Sensitivity	Independent FIT CRC Sensitivity
Index Lesion Size, % (95% CI); n/N		
<5 mm	100.0 (2.5-100.0); 1/1	100.0 (2.5-100.0); 1/1
5–9 mm	100.0 (2.5-100.0); 1/1	100.0 (2.5-100.0); 1/1
10–19 mm	87.5 (47.3-99.7); 7/8	62.5 (24.5-91.5); 5/8
20–29 mm	92.3 (64.0-99.8); 12/13	61.5 (31.6-86.1); 8/13
≥30 mm	96.8 (88.8-99.6); 60/62	72.6 (59.8-83.1); 45/62
Index Lesion Location, % (95% CI); n/N		
Proximal	93.5 (78.6-99.2); 29/31	61.3 (42.2-78.2); 19/31
Distal	93.8 (79.2-99.2); 30/32	78.1 (60.0-90.7); 25/32
Rectal	100.0 (84.6-100.0); 22/22	72.7 (49.8-89.3); 16/22
CRC Stage, % (95% CI); n/N		
I	88.0 (68.8-97.5); 22/25	56.0 (34.9-75.6); 14/25
II	92.9 (66.1-99.8); 13/14	78.6 (49.2-95.3); 11/14
III	100.0 (88.4-100.0); 30/30	73.3 (54.1-87.7); 22/30

CRC Subgroup	Cologuard Plus CRC Sensitivity	Independent FIT CRC Sensitivity
IV	100.0 (73.5-100.0); 12/12	83.3 (51.6-97.9); 10/12
X	100.0 (39.8-100.0); 4/4	75.0 (19.4-99.4); 3/4
Stage I-III combined	94.2 (85.8-98.4); 65/69	68.1 (55.8-78.8); 47/69

Table 16-18: The Cologuard Plus Test APL Sensitivity by Colonoscopy Categories Compared to Independent FIT APL Sensitivity with Confidence Intervals

APL Subgroup	Cologuard Plus APL Sensitivity	Independent FIT APL Sensitivity
Index Lesion Size, % (95% CI); n/N		
<5 mm	33.3 (4.3-77.7); 2/6	0.0 (0.0-45.9); 0/6
5–9 mm	28.2 (18.1-40.1); 20/71	26.8 (16.9-38.6); 19/71
10–19 mm	39.0 (36.6-41.5); 609/1,561	20.5 (18.5-22.6); 320/1,561
20–29 mm	62.6 (55.9-69.0); 139/222	32.4 (26.3-39.0); 72/222
≥30 mm	78.0 (68.6-85.7); 78/100	46.0 (36.0-56.3); 46/100
Index Lesion Location, % (95% CI); n/N		
Proximal	39.3 (36.4-42.2); 440/1,120	15.7 (13.6-18.0); 176/1,120
Distal	48.0 (44.1-51.9); 315/656	35.7 (32.0-39.5); 234/656
Rectal	50.5 (43.1-58.0); 93/184	25.5 (19.4-32.5); 47/184
APL Subcategory, % (95% CI); n/N		
2.1	66.2 (58.3-73.6); 104/157	46.5 (38.5-54.6); 73/157
2.1a	73.6 (64.1-81.7); 78/106	48.1 (38.3-58.0); 51/106
2.1b	51.0 (36.6-65.2); 26/51	43.1 (29.3-57.8); 22/51
2.2	54.8 (50.3-59.2); 269/491	33.2 (29.0-37.6); 163/491
2.3	33.3 (30.5-36.2); 359/1,077	19.5 (17.2-22.0); 210/1,077
2.4	49.4 (42.8-55.9); 116/235	4.7 (2.4-8.2); 11/235

Results for CRC sensitivity, APL sensitivity, and specificity were consistent with the primary and secondary endpoint results in age-weighted estimation based on the age distribution of the US Population, multiple imputation for missing test results, and analysis of all available data.

Age-weighted to the U.S. Population, Category 3-6 specificity was 91.8% (95% CI 91.2-92.4) and Category 6 specificity was 93.8% (95% CI 93.2-94.5).

Table 16-19: The Cologuard Plus Test APL Sensitivity by Colonoscopy Categories, Compared to Independent FIT APL Sensitivity

APL Subgroup	Cologuard Plus n/N	Cologuard Plus Sensitivity	FIT n/N	FIT Sensitivity
APL Subtype[a]				
High-grade dysplasia or ≥10 adenomas, any size	104/157	66.2%	73/157	46.5%
High-grade dysplasia, any size	78/106	73.6%	51/106	48.1%

APL Subgroup	Cologuard Plus n/N	Cologuard Plus Sensitivity	FIT n/N	FIT Sensitivity
≥10 adenomas, any size	26/51	51.0%	22/51	43.1%
Tubulovillous adenoma, any size	269/491	54.8%	163/491	33.2%
Tubular adenoma ≥10 mm	359/1,077	33.3%	210/1,077	19.5%
Sessile serrated lesion with dysplasia (SSLD); Traditional serrated adenoma (TSA), Conventional adenoma with serrated architecture; Sessile serrated lesion; ≥10 mm	116/235	49.4%	11/235	4.7%
APL Location				
Proximal	440/1,120	39.3%	176/1,120	15.7%
Distal	315/656	48.0%	234/656	35.7%
Rectal	93/184	50.5%	47/184	25.5%
Lesion Size				
<5 mm	2/6	33.3%	0/6	0.0%
5–9 mm	20/71	28.2%	19/71	26.8%
10–19 mm	609/1,561	39.0%	320/1,561	20.5%
20–19 mm	139/222	62.6%	72/222	32.4%
≥30 mm	78/100	78.0%	46/100	46.0%
All High-Grade Dysplasia plus any APL				
≥15 mm	433/728	59.5%	235/728	32.3%
≥20 mm	275/425	64.7%	162/425	38.1%

[a] Please refer to Table 16-4: Participant Categorization Based on Histopathologic Diagnosis of the Index Lesion for descriptions of the APL subcategories.

The following baseline characteristics were evaluated for potential association with safety and effectiveness outcomes: sex, age, and race/ethnicity (results presented in Table 16-20

Table 16-20: The Cologuard Plus Test Performance by Subgroup

Subgroup	CRC Sensitivity %; n/N	APL Sensitivity %; n/N	Specificity for Category 3-6 %; n/N
Sex			
Male	95.5%; 42/44	44.1%; 494/1,121	89.8%; 6,928/7,711
Female	95.1%; 39/41	42.2%; 355/841	91.4%; 8,369/9,153
Age			
45–49 years	100.0%; 1/1	28.6%; 4/14	97.8%; 268/274
50–54 years	100.0%; 2/2	32.5%; 37/114	96.1%; 1,363/1,419
55–59 years	100.0%; 17/17	41.3%; 181/438	92.5%; 3,788/4,095

Subgroup	CRC Sensitivity %; n/N	APL Sensitivity %; n/N	Specificity for Category 3-6 %; n/N
60–64 years	94.4%; 17/18	39.0%; 150/385	91.1%; 2,867/3,148
65–69 years	93.1%; 27/29	46.4%; 289/623	89.4%; 4,325/4,836
70–74 years	92.3%; 12/13	47.9%; 134/280	87.4%; 1,924/2,201
≥75 years	100.0%; 5/5	50.0%; 54/108	85.5%; 762/891
Race/Ethnicity			
White, Not Hispanic or Latino	94.7%; 54/57	46.4%; 597/1,287	88.9%; 8,842/9,942
Hispanic or Latino	100.0%; 11/11	43.1%; 125/290	92.8%; 2,593/2,793
Black or African American, Not Hispanic or Latino	90.9%; 10/11	38.0%; 98/258	92.3%; 2,089/2,263
Asian, Not Hispanic or Latino	100.0%; 4/4	20.0%; 20/100	95.1%; 1,522/1,600
American Indian or Alaskan Native, Not Hispanic or Latino	--------	42.9%; 3/7	90.0%; 54/60
Native Hawaiian or Other Pacific Islander, Not Hispanic or Latino	--------	25.0%; 1/4	94.7%; 18/19
Multiracial, Not Hispanic or Latino	--------	25.0%; 1/4	95.9%; 70/73
Other, Not Hispanic or Latino	100.0%; 2/2	33.3%; 3/9	96.2%; 101/105

The specificity of the Cologuard Plus test remained high across guideline-recommended ages for CRC screening.

- Among participants 45–54 years of age, Cologuard Plus specificity was comparable to that of FIT, with Category 3–6 specificities of 96.3% and 95.7%, respectively, and Category 6 specificities of 96.8% and 96.3%, respectively.
- Cologuard Plus specificity remained high in participants aged 55-64, with a Category 3–6 specificity of 91.9% and a Category 6 specificity of 93.6%.
- Specificity in participants aged 70–74 remained relatively high at 87.4% for Category 3–6 and 89.9% for Category 6.

There were no adverse events related to the stool collection procedure.

Overall, the results of the BLUE-C study demonstrate the safety and effectiveness of the Cologuard Plus test as a non-invasive, stool-based method for use in average risk adults for colorectal cancer screening.

17 Abbreviations Used

Table 17-1: Definitions of Abbreviations Used.

Abbreviation	Definition
ANSI	American National Standards Institute
ADL	Allowable Deviation from Linearity
APL	Advanced Precancerous Lesion
AvgMDM	The median weighted average of the reference-normalized standardized methylation marker DNA concentrations. Used along with Hemoglobin concentration to calculate the Cologuard Plus score.
Cp	Crossing Point (the cycle at which there is a significant increase in fluorescence)
CRC	Colorectal Cancer
DHHC	Enzyme Palmitoyl transferase named after a conserved sequence motif found in its protein sequence
FDFH	First-degree Family History
FIT	Fecal Immunochemical Test
FPF	False Positive Fraction
HRP	Horseradish Peroxidase
IBD	Inflammatory Bowel Disease
IPA	Isopropyl Alcohol, Isopropanol
IU	Intended Use
LASS4	Ceramide Synthase 4
LLOQ	Lower Limit of Quantitation
LOB	Limit of Blank
LOD	Limit of Detection
LOQ	Limit of Quantitation
LQAS	Long-probe Quantitative Amplified Signal assay
LRRC4	Leucine Rich Repeat Containing 4
MDM	Methylated DNA Marker, indicative of cancer
NCI	National Cancer Institute
NTC	No template control consisting of Poly(A) diluent at 10 ng/µL
PPP2R5C	Protein Phosphatase 2 Regulatory Subunit B'G
PVPP	Poly(vinylpolypyrrolidone)
QS5 Dx	Quant Studio 5 Dx
SEER	Surveillance, Epidemiology, and End Results program from NCI
SLIB	Supplemental Lot Information Barcode
sDNA	Stool DNA
ZDHHC1	Zinc Finger DHHC-Type Containing 1

18 Key Symbols Used

Table 18-1: Symbols Used

Symbol	Description
	Consult Instructions for Use
	In vitro Diagnostic Device
	Catalog Number
	Part Number
	Indicates upper and lower temperature limits for storage
	Light sensitive, Protect from light.
	Corrosive
	Harmful
	Manufacturer

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20 Contact Information

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1 Intended Use and Indications for Use

The Cologuard Plus™ test is a qualitative in vitro diagnostic test intended for the detection of colorectal neoplasia-associated DNA markers and for the presence of occult hemoglobin in human stool. The Cologuard Plus test is performed on samples collected using the Cologuard Plus Collection Kit. A positive result may indicate the presence of colorectal cancer (CRC) or advanced precancerous lesions (APL) and should be followed by colonoscopy. The Cologuard Plus test is indicated to screen adults 45 years or older, who are at average risk for CRC. The Cologuard Plus test is not a replacement for diagnostic colonoscopy or surveillance colonoscopy in high-risk individuals.

The Cologuard Plus test is performed at Exact Sciences, Madison, WI.

2 Contraindications

The Cologuard Plus test is not indicated for use in patients who have the following:

- A personal history of CRC or APLs.
- A positive result from another CRC screening method within the last 6 months, or:
  - 12 months for a fecal occult blood test (FOBT) or a fecal immunochemical test (FIT)
  - 36 months for a FIT-DNA test
- A family history of CRC, defined as having a first-degree relative (parent, sibling, or child) with a CRC diagnosis at any age.
- Personal history of any of the following high-risk conditions for CRC:
  - A diagnosis of Inflammatory Bowel Disease (Chronic Ulcerative Colitis, Crohn's Disease).
  - A diagnosis of a relevant familial (hereditary) cancer syndrome or other polyposis syndrome, including but not limited to: Familial adenomatous polyposis (FAP or Gardner's), Hereditary non-polyposis colorectal cancer syndrome (HNPCC or Lynch), Peutz-Jeghers, MYH-Associated Polyposis (MAP), Turcot's (or Crail's), Cowden's, Juvenile Polyposis, Cronkhite-Canada, Neurofibromatosis, or Serrated Polyposis.

3 Warnings and Precautions

- Patients should not provide a sample if they are experiencing diarrhea or have known blood in their urine or stool (e.g., from bleeding hemorrhoids, bleeding cuts or wounds on their hands, rectal bleeding, or menstrual bleeding). Unexpected bleeding should be discussed with your healthcare provider.
- Reference national guidelines for the recommended screening ages for colorectal cancer.[4] The decision to screen persons over the age of 75 should be made on an individualized basis in consultation with your healthcare provider. Cologuard Plus test results should be interpreted with caution in older patients as the rate of false positive results increases with age.
- The Cologuard Plus test may produce false negative or false positive results. A false positive result occurs when the Cologuard Plus test produces a positive result, even though a colonoscopy will not find CRC or APL. A false negative result occurs when the Cologuard Plus test does not detect an APL or CRC even when a colonoscopy identifies either of these findings.
  - Out of every 100 patients testing positive, approximately 3 patients will have CRC, 34 patients will have APL, 33 will have a non-advanced adenoma, and 30 will have no neoplastic findings.
  - Out of every 10,000 patients testing negative, approximately 2 will be falsely assured that they do not have CRC. Out of every 100 patients testing negative, approximately 7 patients will be falsely assured that they do not have APL.
- A negative Cologuard Plus test result does not guarantee the absence of CRC or APL. Patients with a negative Cologuard Plus test result should continue participating in colorectal cancer screening programs, at the appropriate guideline recommended intervals.
- The performance of the Cologuard Plus test has been established in a cross-sectional study (i.e., single point in time). Programmatic performance of the Cologuard Plus test (i.e., benefits and risks with repeated testing over an established period of time) has not been studied. Non-inferiority or superiority of the Cologuard Plus test's programmatic sensitivity as compared to other recommended screening methods for CRC and APL has not been established.
- To ensure the integrity of the sample, the laboratory must receive the patient specimens within 144 hours of collection. Patients should send stool samples to the laboratory according to the instructions included in the Cologuard Plus Collection Kit.
- Patients should be advised of the caution listed in the Cologuard Plus Collection Kit instructions. Patients should NOT drink the preservative liquid.
- The risks related to using the Cologuard Plus Collection Kit are low, with no serious adverse events reported among people in a clinical trial. Patients

should be careful when opening and closing the lids to avoid the risk of hand strain. Fecal samples should be treated as if they are potentially infectious.

Rx Only

4 The Cologuard Plus Test Overview

4.1 Summary of the Cologuard Plus Test Performance

The Cologuard Plus test demonstrated 95.3% CRC sensitivity, 43.3% APL sensitivity, and 90.7% specificity among participants with neither CRC nor APL.

4.2 Patient Samples for the Cologuard Plus Test

Patients are not required to undergo bowel preparation or follow dietary or medication restrictions in order to complete the test. Patients follow the detailed instructions received with the Cologuard Plus Collection Kit, consisting of a container for collection of stool for DNA testing and a separate Tube and Probe for collection of stool for hemoglobin testing. Both of these stool samples are required to obtain a Cologuard Plus test result. Samples are sent to Exact Sciences Laboratories for processing and testing.

4.3 The Cologuard Plus Test Patient Navigation Program

The Cologuard Plus test includes a patient support program. Customer Care Specialists are available 24 hours a day, 7 days a week to communicate with patients in over 240 languages about the Cologuard Plus test sample collection or return questions. Representatives are also available to answer billing or reimbursement questions. Exact Sciences Laboratories sends patients reminders about completing the Cologuard Plus Collection Kit. This program also provides tracking for healthcare providers so they can measure and monitor patient adherence to Cologuard Plus test screening.

5 Colorectal Cancer Overview

Colorectal cancer (CRC) is the second leading cause of cancer death among men and women in the United States, with more than 153,000 individuals diagnosed annually.[1] One in 24 Americans will suffer from CRC during their lifetime.[10] Early detection by screening has been shown to reduce CRC mortality.[2-8] Based on increasing incidence of CRC in younger adults, current guidelines for CRC screening in the average-risk population recommend initiation of screening at age 45.[3-6,] The 2021 US Preventive Services Task Force (USPSTF) recommendation concludes that initiating colorectal cancer screening at age 45 provides moderate certainty of moderate net benefit,[4] whereas the 2018 guideline update from the American Cancer Society (ACS) gave a qualified recommendation to initiate screening at age 45 in average risk individuals.[3] In addition, the American College of Gastroenterology (ACG) and US Multi-Society Task Force (MSTF) updated their CRC screening guidelines in 2021 to recommend initiation of screening at age 45 for average risk individuals.[5,6]

Approximately 40% of adults 45 years of age or older are not current with recommended CRC screening.[1] Less than half of adults 50-54 years of age and only 17.8% of adults ages 40-49 report recent screening for CRC.[3]

Detection of potentially pre-malignant lesions, also known as advanced precancerous lesions, is essential for CRC prevention. APLs include any size adenomas with carcinoma in situ or high-grade dysplasia (HGD), adenomas with villous growth patterns (≥25%), adenomas ≥1.0 cm in size or serrated lesions ≥1.0 cm in size.[7-9] Serrated lesions (polyps and sessile serrated adenomas) are typically found in the proximal colon, occur more frequently in the elderly, are often flat and inconspicuous endoscopically, and may have a more aggressive natural history than classic colorectal adenomas.[9]

6 Device Description

The Cologuard Plus test is an in vitro diagnostic device designed to analyze a patient's stool for the presence of DNA and hemoglobin markers which may indicate the presence of CRC or APL. Specifically, two independent categories of biomarkers are targeted and provide an additive association for the detection of CRC and pre-malignant neoplasms. The combined result/composite score gives a qualitative result, Positive (abnormal) or Negative (normal) which is associated with increased or decreased likelihood of CRC and APL, respectively.

The first category of biomarkers detects epigenetic DNA changes characterized by aberrant gene promoter region methylation. The specific methylated gene targets include ceramide synthase 4 gene *(LASS4)*, leucine-rich repeat-containing protein 4 gene *(LRRC4)*, and protein phosphatase 2 regulatory subunit B' gene *(PPP2R5C)*. *LASS4, LRRC4,* and *PPP2R5C* have been shown to be hypermethylated in colorectal cancer.[12-14] The Cologuard Plus procedure incorporates bisulfite conversion of non-methylated cytosine residues to uracil

in the DNA sequence to enable sensitive detection of hypermethylated *LASS4, LRRC4, and PPP2R5C*. The second category of biomarker is non-DNA based and detects hemoglobin, which can be associated with colonic bleeding. Results from the molecular and hemoglobin assays are integrated by the laboratory analysis to determine a Positive or Negative reportable result or No Result Obtained.[16]

6.1 Assay Technology

The patient stool samples are processed at Exact Sciences Laboratories to isolate the DNA for testing. Amplification and detection of the hypermethylated target DNA *LASS4*, *LRRC4*, *PPP2R5C*, and *ZDHHC1* (a reference gene) is performed by incorporating bisulfite conversion of non-methylated cytosine residues to uracil in the DNA sequence to enable sensitive detection of the hypermethylated target DNA using the Long-probe Quantitative Amplified Signal (LQAS) technology, which combines real-time PCR and invasive cleavage to perform allele-specific amplification and detection of methylated target DNA in the molecular assay. In a parallel workflow, the hemoglobin stool sample is prepared and analyzed in a quantitative Enzyme-Linked Immunosorbent Assay (ELISA) that determines the concentration of hemoglobin in the sample.

Run control samples for both the DNA assay and hemoglobin assay are tested along with patient samples to show that the process has been performed appropriately. Results from the DNA and hemoglobin assays are integrated during analysis to determine a Positive or Negative reportable result or No Result Obtained.

7 Clinical Study: Multi-Target Stool DNA Test for Colorectal Cancer Screening: BLUE-C

7.1 Overview

The Cologuard Plus test was the subject of a prospective, cross-sectional, multi-center, pivotal trial, Multi-Target Stool DNA Test for Colorectal Cancer Screening: BLUE-C Study, ("BLUE-C" or "the study").[17] A total of 26,758 participants were enrolled from 186 sites, including both colonoscopy centers and primary care sites, in the United States. The results of the study demonstrated the safety and effectiveness of the Cologuard Plus test as a screening test for the detection of markers associated with the presence of CRC and APL. The Cologuard Plus test demonstrated 95.3% CRC sensitivity and 90.7% specificity (specificity in this study excludes CRC and APL), using colonoscopy with histopathological confirmation as the reference method. These results met the protocol-specified criteria for primary performance measures and study success. The study further compared CRC and APL detection by the Cologuard Plus test to a commercially available fecal immunochemical test (OC-Auto® Micro 80, Polymedco, Inc.) ("FIT"), demonstrating superiority for CRC ($p<0.0001$) and APL ($p<0.0001$) sensitivity.

7.2 Study Design

The study was designed to enroll male or female participants, who were at average risk for development of CRC and asymptomatic for gastrointestinal symptoms warranting diagnostic colonoscopy. Subject enrollment was enriched toward a slightly older population to increase the point prevalence of colorectal cancer in this study. Enrollment was also focused on colonoscopy-naïve participants aged ≥55 years because of the higher prevalence of CRC in this population. Forty-eight percent of participants in the actual study population were 65 years of age or older.

In addition to the study enrollment eligibility criteria, the primary analysis population excluded participants with a first-degree relative with CRC at any age, as well as participants under the age of 45 years.

Trial participants provided a stool sample and subsequently underwent colonoscopy within 180 days of stool collection. Participants collected stool samples for the Cologuard Plus test and independent FIT testing at home. Participants then underwent colonoscopy per standard of care. Participants and physicians remained blinded to the results of the Cologuard Plus test and the FIT. Results from the Cologuard Plus test and FIT were compared to the results of the colonoscopy examination and histopathologic diagnosis of all significant lesions either biopsied or removed.

Histopathological results from biopsied tissue or excised lesions were categorized based on the most clinically significant lesion present (i.e. the index lesion) by a central pathologist according to the pre- specified standards outlined in Table 7-1. Participants with no findings on colonoscopy and no biopsy(ies) taken were categorized as 6.2. Sensitivity calculations were performed using positive findings in categories 1 and 2 while specificity was calculated using categories 3 through 6 (all findings excluding CRC and APL). Stages of CRC were recorded based on the American Joint Committee on Cancer (AJCC) Staging System, 8th edition.[15]

Table 7-1: Participant Categorization Based on Histopathologic Diagnosis of the Index Lesion

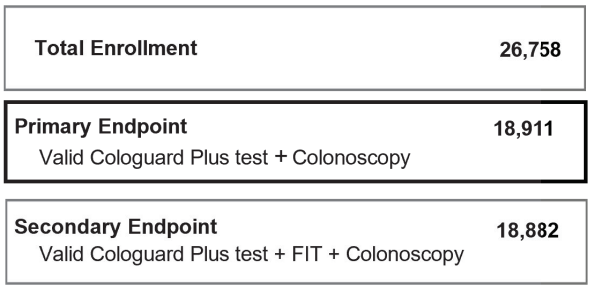
Category	Description
1	Stage I-IV colorectal cancer, any size
2	Advanced Precancerous Lesions (APL), including the following subcategories:
2.1	High-grade dysplasia or ≥10 adenomas, any size
2.1a	High-grade dysplasia, any size
2.1b	≥10 adenomas, any size
2.2	Tubulovillous adenoma, any size
2.3	Tubular adenoma, ≥10 mm
2.4	Sessile serrated lesion with dysplasia (SSLD); Traditional serrated adenoma (TSA), Conventional adenoma with serrated architecture; Sessile serrated lesion; ≥10 mm
3	3-9 adenomas or sessile serrated lesions, <10 mm, non-advanced
4	1-2 adenomas or sessile serrated lesions, 5-9 mm, non-advanced
5	1-2 adenomas or sessile serrated lesions, <5 mm, non-advanced
6	Negative: no adenocarcinoma of the colorectum, no adenomas or SSA/SSP
6.1	Hyperplastic polyps or non-neoplastic lesions
6.2	No lesions on colonoscopy
X	Index Lesion could not be categorized because tissue/report was lost/not provided or histopathological diagnosis could not be determined.

7.3 Demographic and Baseline Characteristics

Study enrollment and population demographics are summarized in Figure 7-1.

Of the total 26,758 participants enrolled in the study, 18,911 participants with colonoscopy and Cologuard Plus test data were included in the primary analysis population. This population includes 85 participants with CRC. Analyses conducted to assess the impact of enrichment strategies and evaluate potential bias associated with participants excluded from the analysis population yielded results consistent with the primary analyses.

The average age of participants was 63.0 years, and there was a slightly higher percentage of female participants 53.1% (10,035/18,911) as compared with male participants 46.9% (8,876/18,911). The majority of participants were White, not Hispanic or Latino 59.70% (11,286/18,911). Among the minorities in the study population, there were Hispanic or Latino 16.4% (3,094/18,911); Black or African American, not Hispanic or Latino 13.4% (2,532/18,911); and Asian, not Hispanic or Latino 9.0% (1,704/18,911). Other race and ethnicity categories with smaller enrollment distribution are included in the figure below. Average Body Mass Index (BMI) was 29.5 kg/m^2 and the majority of participants never smoked 63.6% (12,019/18,911).



Demographics		
Age (Years)	Average	63.0
	Range	45-86
Sex	Male	46.9%
	Female	53.1%
BMI (kg/m²)	Average	29.5
	Range	13.0-69.2
Ethnicity/ Race	Hispanic/Latino	16.4%
	Not Hispanic or Latino	
	White	59.7%
	Black	13.4%
	Asian	9.0%
	Amer. Ind/Alaskan Native	0.4%
	Native Hawaiian or Other Pacific Islander	0.1%
	Multiracial	0.4%
	Other	0.6%
Smoking History	Never Smoked	63.6%
	Former Smoker	24.4%
	Smoker	12.1%

Figure 7-1: Clinical Study Demographics

7.4 Clinical Performance Measures

The primary and secondary performance measures for the clinical study are summarized in Table 7-2 below. The primary performance measures were the sensitivity of the Cologuard Plus test for CRC and specificity among participants not having CRC or APL, using colonoscopy with histopathology as the reference method. The primary analysis required the one-sided 97.5% exact confidence interval (CI) to be greater than the 75% null hypothesis value for the sensitivity of the Cologuard Plus test for CRC. The specificity analysis required the one-sided 97.5% exact CI to be greater than the 85.9% null hypothesis value.

For the secondary performance measures, evaluating the Cologuard Plus test sensitivity for APL detection required the one-sided 97.5% exact CI to exceed the null hypothesis value of 38.9%; evaluating specificity of the Cologuard Plus test for no colorectal neoplastic findings required the one- sided 97.5% exact CI to exceed the null hypothesis value of 87.5%. The head-to-head comparisons with FIT were performed using exact McNemar's tests for paired proportions at the one-sided 2.5% significance level.

Table 7-2: Clinical Study Primary and Secondary Performance Measures

Primary Performance Measures	• Determine the sensitivity of the Cologuard Plus test for CRC detection. • Determine the specificity of the Cologuard Plus test for Categories 3–6 (no APL or CRC).
Secondary Performance Measures	• Determine the sensitivity of the Cologuard Plus test for APL detection. • Compare the Cologuard Plus test sensitivities for CRC and APL detection to FIT. • Evaluate the specificity of the Cologuard Plus test for participants with no colorectal neoplastic findings (Category 6).

7.5 Summary of Clinical Study Results

Results from the clinical study, summarized in Table 7-3, demonstrated that the Cologuard Plus test successfully met both the primary and secondary performance measures of the study, establishing a clinically meaningful sensitivity for CRC and specificity. Sensitivity of the Cologuard Plus test for CRC was 95.3% (81/85) with a one-sided 97.5% lower confidence bound of 88.4%, above the pre-specified threshold for study success. Sensitivity of the Cologuard Plus test for APL detection was 43.3%, with a one-sided 97.5% lower confidence bound of 41.1%, exceeding the protocol-specified threshold for study success. In addition, the Cologuard Plus test successfully demonstrated a

clinically meaningful specificity according to the protocol-specified criteria. The specificity of the Cologuard Plus test for Category 3–6 was 90.7%, with a one-sided 97.5% lower confidence bound of 90.3%, above the prespecified threshold for study success. For Category 6, the specificity of the Cologuard Plus test was 92.7% with a one- sided 97.5% lower confidence bound of 92.2%, above the pre-specified threshold for study success.

Table 7-3: Summary of the Cologuard Plus Test Performance

Colonoscopy/Histo-pathology	Primary Effectiveness Population
	Sensitivity %, (95% CI) (n detected/N)
CRC	95.3 (88.4, 98.7) (81/85)
APL	43.3 (41.1, 45.5) (849/1,962)
	Specificity % (95% CI) (n negative/N)
Category 3–6	90.7 (90.3, 91.1) (15,297/16,864)
No colorectal neoplasia (Category 6)	92.7 (92.2, 93.2) (9,609/10,361)

The clinical study also compared the Cologuard Plus test sensitivity and specificity to an independent commercially available FIT. Results demonstrated superiority of the Cologuard Plus test to FIT for sensitivity in detecting CRC. Sensitivity for CRC was greater for the Cologuard Plus test compared to FIT (95.3% vs. 70.6%, respectively, exact McNemar p<0.0001). The Cologuard Plus test identified 21 of 25 (84.0%) CRC cases that were missed by FIT, while FIT did not identify any cancer cases that were not identified by the Cologuard Plus test.

Sensitivity for APL was greater for the Cologuard Plus test compared to FIT (43.3% vs. 23.3%, respectively, exact McNemar p<0.0001). The Cologuard Plus test identified 506 of 1,503 (33.7%) APL cases missed by FIT, while FIT identified 115 of 1,112 (10.3%) APL cases missed by the Cologuard Plus test.

The specificity for Category 3–6 of the Cologuard Plus test was 90.7% and of FIT was 94.8%. These specificity measures excluded CRC and APL for both tests.

The positive and negative predictive values (PPV and NPV) of the Cologuard Plus test were calculated. The PPV of the Cologuard Plus test was 3.2% (81/2,497) for CRC and 34.0% (849/2,497) for APL. Among the participants with a positive Cologuard Plus test result, 69.9% (1,745/2,497) were found to have a CRC, APL, or non-advanced adenoma. The NPV of the Cologuard Plus test was 99.98% (16,410/16,414) for CRC, and 93.2% (15,297/16,414) for advanced neoplasia (CRC or APL). Clinical results show that a negative patient result for the Cologuard Plus test gives 99.98% assurance that the patient does not have CRC and a 93.2% chance that the patient does not have any CRC or APL.

Table 7-4: Positive and Negative Predictive Values: Index Lesion Categorization by the Cologuard Plus Test Result

Index Lesion Categorization	Positive Predictive Value (PPV), % (95% CI); n/N positive test results	1-Negative Predictive Value (1-NPV), % (95% CI); n/N negative test results
CRC (n=85)	3.2 (2.6-4.0); 81/2,497	0.02 (0.01-0.06); 4/16,414
APL (n=1,962)	34.0 (32.1-35.9); 849/2,497	6.8 (6.4-7.2); 1,113/16,414
Category 3–5 (n=6,503)	32.6 (30.8-34.5); 815/2,497	34.7 (33.9-35.4); 5,688/16,414
Category 6 (n=10,361)	30.1 (28.3-32.0); 752/2,497	58.5 (57.8-59.3); 9,609/16,414

Age-weighted to the U.S. Population, Category 3-6 specificity was 91.8% (95% CI 91.2-92.4) and Category 6 specificity was 93.8% (95% CI 93.2-94.5).

7.6 The Cologuard Plus Test and FIT Sensitivity by Lesion Subgroups

The Cologuard Plus test demonstrated high sensitivity for detection of lesions and polyps which historically have been difficult to capture with FIT, including early-stage CRC, proximal lesions, and higher risk precancerous lesions. The Cologuard Plus test demonstrated a numerically greater sensitivity than FIT for detection of CRC and APLs across lesion subgroups. Sensitivity results are summarized in Table 7-5 and Table 7-6.

The CRC sensitivity of the Cologuard Plus across all cancer stages was as follows: 88.0% (22/25) in Stage I, 92.9% (13/14) in Stage II, 100% (30/30) in Stage III, and 100.0% (12/12) in Stage IV (See Table 7-5). In the curative stages, Stages I–III combined, the Cologuard Plus test sensitivity was 94.2% (65/69). CRC sensitivity for independent FIT was substantially lower at 56.0% (14/25) in Stage I, 78.6% (11/14) in Stage II, 73.3%

(22/30) in Stage III, 83.3% (10/12) in Stage IV, and 68.1% (47/69) in Stage I–III combined.

The CRC sensitivity of the Cologuard Plus test was generally consistent across lesion sizes and locations: 87.5% (7/8) in CRCs 10–19 mm, 92.3% (12/13) in CRCs 20– 29mm, 96.8% (60/62) in CRCs ≥30 mm, 93.5% (29/31) in proximal CRCs, 93.8% (30/32) in distal CRCs, and 100% (22/22) in rectal cancers. The CRC sensitivity of the independent FIT was substantially lower: 62.5% (5/8) in CRCs 10-19 mm, 61.5% (8/13) in CRCs 20-29 mm, 72.6% (45/62) in CRCs ≥30 mm, 61.3% (19/31) in proximal CRCs, 78.1% (25/32) in distal CRCs, and 72.7% (16/22) in rectal cancers.

APL sensitivity of the Cologuard Plus test, delineated in Table 7-6, increased with lesion size, from 33.3% (2/6) in APLs <5 mm, 28.2% (20/71) in APLs 5-9 mm, 39% (609/1,561) in APLs 10-19 mm, 62.6% (139/222) in APLs 20-29 mm, and 78.0% (78/100) in APLs ≥30 mm. The independent FIT APL sensitivity was substantially lower for each size and location, at 0% (0/6) in APLs <5 mm, 26.8% (19/71) in APLs 5-9 mm, 20.5% (320/1,561) in APLs 10-19 mm, 32.4% (72/222) in APLs 20-29 mm, and 46.0% (46/100) in APLs ≥ 30mm. APL sensitivity of the Cologuard Plus test by location was 39.3% (440/1,120) in proximal APLs, 48.0% (315/656) in distal APLs, and 50.5% (93/184) in rectal APLs. FIT APL sensitivity by location was also substantially lower: 15.7% (176/1,120) in proximal APLs, 35.7% (234/656) in distal APLs, and 25.5% (47/184) in rectal APLs.

Cologuard Plus test APL sensitivity was 73.6% (78/106) for high grade dysplasia, 54.8% (269/491) for tubulovillous adenomas of any size, 33.3% (359/1,077) for tubular adenomas ≥10 mm, and 49.4% (116/235) for serrated lesions≥10 mm. In the higher risk APL combination of all high-grade dysplasia plus any APL ≥15 mm or ≥20 mm, Cologuard Plus test sensitivity was 59.5% (433/728) and 64.7% (275/425) respectively.

FIT sensitivity was 48.1% (51/106) for high grade dysplasia, 33.2% (163/491) for tubulovillous adenomas of any size, 19.5% (210/1,077) for tubular adenomas ≥10 mm, and 4.7% (11/235) for serrated lesions ≥10 mm. In the higher risk APL combination of all high-grade dysplasia plus any APL ≥15 mm or ≥20 mm, independent FIT sensitivity was 32.3% (235/728) and 38.1% (162/425) respectively. As a preplanned exploratory analysis, APL sensitivity was compared between the Cologuard Plus test and FIT for each APL subcategory; for each subcategory, the Cologuard Plus test sensitivity was superior to FIT ($p<0.0001$ for each by McNemar test).

Table 7-5: The Cologuard Plus test CRC Sensitivity by Colonoscopy Categories, Compared to Independent FIT CRC Sensitivity

Subgroup	Cologuard Plus n/N	Cologuard Plus Sensitivity	FIT n/N	FIT Sensitivity
Cancer Stage[15]				
I	22/25	88.0%	14/25	56.0%
II	13/14	92.9%	11/14	78.6%
III	30/30	100.0%	22/30	73.3%
IV	12/12	100.0%	10/12	83.3%
X	4/4	100.0%	3/4	75.0%
Stage I–III combined	65/69	94.2%	47/69	68.1%
Cancer Size				
<5 mm	1/1	100.0%	1/1	100.0%
5–9 mm	1/1	100.0%	1/1	100.0%
10–19 mm	7/8	87.5%	5/8	62.5%
20–29 mm	12/13	92.3%	8/13	61.5%
≥30 mm	60/62	96.8%	45/62	72.6%
Cancer Location				
Proximal	29/31	93.5%	19/31	61.3%
Distal	30/32	93.8%	25/32	78.1%

Subgroup	Cologuard Plus n/N	Cologuard Plus Sensitivity	FIT n/N	FIT Sensitivity
Rectal	22/22	100.0%	16/22	72.7%

Table 7-6: The Cologuard Plus test APL Sensitivity by Colonoscopy Categories, Compared to Independent FIT APL Sensitivity

APL Subgroup	Cologuard Plus n/N	Cologuard Plus Sensitivity	FIT n/N	FIT Sensitivity
APL Subtype[a]				
High-grade dysplasia or ≥10 adenomas, any size	104/157	66.2%	73/157	46.5%
High-grade dysplasia, any size	78/106	73.6%	51/106	48.1%
≥10 adenomas, any size	26/51	51.0%	22/51	43.1%
Tubulovillous adenoma, any size	269/491	54.8%	163/491	33.2%
Tubular adenoma≥10 mm	359/1,077	33.3%	210/1,077	19.5%
Sessile serrated lesion with dysplasia (SSLD); Traditional serrated adenoma (TSA), Conventional adenoma with serrated architecture; Sessile serrated lesion; ≥10 mm	116/235	49.4%	11/235	4.7%
APL Location				
Proximal	440/1,120	39.3%	176/1,120	15.7%
Distal	315/656	48.0%	234/656	35.7%
Rectal	93/184	50.5%	47/184	25.5%
Lesion Size				
<5 mm	2/6	33.3%	0/6	0.0%
5–9 mm	20/71	28.2%	19/71	26.8%
10–19 mm	609/1,561	39.0%	320/1,561	20.5%
20–19 mm	139/222	62.6%	72/222	32.4%
≥30 mm	78/100	78.0%	46/100	46.0%
All High-Grade Dysplasia plus any APL				
≥15 mm	433/728	59.5%	235/728	32.3%
≥20 mm	275/425	64.7%	162/425	38.1%

[a] Please refer to Table 7-1: Participant Categorization Based on Histopathologic Diagnosis of the Index Lesion for descriptions of the APL subcategories.

7.7 The Cologuard Plus Test Subgroup Analysis

Please note that the clinical study was not designed or powered to evaluate subgroups and subgroup analyses should be interpreted with that in mind. The clinical study results summarized in Table 7-7 below were analyzed across demographic subgroups. CRC sensitivity was greater than 90% for each age range; sex, at 95.5% (42/44) in males and 95.1% (39/41) in females; and race/ethnicity, at 94.7% (54/57) in White, not Hispanic or Latino 100% (11/11) in Hispanic or Latino, 90.9% (10/11) in Black, not Hispanic or Latino, and 100% (4/4) in Asian participants.

APL sensitivity increased with age, from 28.6% (4/14) for ages 45-49, 32.5% (37/114) for ages 50-54, 41.3% (181/438) for ages 55-59, 39.0% (150/385) for ages 60-64, 46.4% (289/623) for ages 65-69, 47.9% (134/280) for ages 70-74, and 50.0% (54/108) for ages greater than 75. APL sensitivity was 44.1% (494/1,121) in males and 42.2% (355/841) in females, and 46.4% (597/1,287) in White, not Hispanic or Latino, 43.1% (125/290) in Hispanic or Latino, 38.0% (98/258) in Black, not Hispanic or Latino, and 20.0% (20/100) in Asian participants.

Specificity for Category 3–6 of the Cologuard Plus test was high in the younger age groups and remained above 90% through age 64. Specificity was 97.8% (268/274) in participants aged 45-49 years, 96.1% (1,363/1,419) in ages 50-54, 87.4% (1,924/2,201) in ages 70-74, and 85.5% (762/891) in age 75 and older. By sex, specificity was 89.8% (6,928/7,711) in males and 91.4% (8,369/9,153) in females. Specificity of the Cologuard Plus test was 88.9% (8,842/9,942) in non-Hispanic or Latino White, 92.8% (2,593/2,793) in Hispanic or Latino, 92.3% (2,089/2,263) in non-Hispanic or Latino Black, and 95.1% (1,522/1,600) in Asian participants.

Table 7-7: The Cologuard Plus Test Performance by Subgroup

Subgroup	CRC Sensitivity %; n/N	APL Sensitivity %; n/N	Specificity for Category 3-6 %; n/N
Sex			
Male	95.5%; 42/44	44.1%; 494/1,121	89.8%; 6,928/7,711
Female	95.1%; 39/41	42.2%; 355/841	91.4%; 8,369/9,153
Age			
45–49 years	100.0%; 1/1	28.6%; 4/14	97.8%; 268/274
50–54 years	100.0%; 2/2	32.5%; 37/114	96.1%; 1,363/1,419
55–59 years	100.0%; 17/17	41.3%; 181/438	92.5%; 3,788/4,095
60–64 years	94.4%; 17/18	39.0%; 150/385	91.1%; 2,867/3,148
65–69 years	93.1%; 27/29	46.4%; 289/623	89.4%; 4,325/4,836
70–74 years	92.3%; 12/13	47.9%; 134/280	87.4%; 1,924/2,201
≥75 years	100.0%; 5/5	50.0%; 54/108	85.5%; 762/891
Race/Ethnicity			
White, Not Hispanic or Latino	94.7%; 54/57	46.4%; 597/1,287	88.9%; 8,842/9,942
Hispanic or Latino	100.0 %; 11/11	43.1%; 125/290	92.8%; 2,593/2,793
Black or African American, Not Hispanic or Latino	90.9%; 10/11	38.0%; 98/258	92.3%; 2,089/2,263
Asian, Not Hispanic or Latino	100.0%; 4/4	20.0%; 20/100	95.1%; 1,522/1,600
American Indian or Alaskan Native, Not Hispanic or Latino	--------	42.9%; 3/7	90.0%; 54/60
Native Hawaiian or Other Pacific Islander, Not Hispanic or Latino	--------	25.0%; 1/4	94.7%; 18/19
Multiracial, Not Hispanic or Latino	--------	25.0%; 1/4	95.9%; 70/73
Other, Not Hispanic or Latino	100.0%; 2/2	33.3%; 3/9	96.2%; 101/105

7.8 Cross-Reactivity

The potential for cross-reactivity with non-colorectal cancers and inflammatory conditions was evaluated using sample specimens collected from subjects with 12 cancer and disease groups other than colorectal cancer (CRC). The table below indicates the final number of cancer or disease patient samples that were tested.

The false positive fraction (FPF) of test results was calculated as a point estimate and a two-sided 95% confidence interval for each disease group. Each FPF was compared to the estimated FPF for the general intended use (IU) population. The disease groups of lung cancer, esophageal cancer, and inflammatory bowel disease did not overlap the estimated FPF for the general IU population. The other nine groups had observed positive test results rates that are consistent with the FPF for the overall assay.

For the assay specificity analysis, the total number of positive calls per 10,000 patients was estimated to be 8.1 to 9.0 with the inclusion of IBD and 7.7 to 8.0 without, as shown in the following table. This was considered a negligible effect on the Cologuard Plus test positivity.

Table 7-8: Cancers and Diseases Tested for Cross-Reactivity

No.	Cancer or Disease[a]	No. of Valid Samples Tested	Incidence per 10,000 population[b]	% Positivity of Cologuard Plus Result	No. Positive Cologuard Plus Calls in 10,000 Patients
1	Autoimmune Disease[c] (individual disease not specified)	29	3.2–5.4	13.8	0.4–0.7
2	Bladder Cancer	5	1.8	20.0	0.4
3	Breast Cancer	35	12.6	11.4	1.4
4	Esophageal Cancer	11	0.4	36.4	0.1
5	Gynecologic Cancer (i.e., endometrial cancer, vulvar melanoma, and ovarian cancer	41	3.8	4.9	0.2
6	Hepatic Cancer (i.e., liver and bile duct cancer)	5	0.9	20.0	0.2
7	Inflammatory Bowel Disease[c]	30	1.5–3.9	26.7	0.4–1.0
8	Kidney/Renal Pelvis Cancer	20	1.7	10.0	0.2
9	Lung Cancer	30	5.0	33.3	1.7
10	Pancreatic Cancer	13	1.3	15.4	0.2
11	Prostate Cancer	35	11.3	22.9	2.6
12	Stomach Cancer	5	0.7	40.0	0.3
Total (with IBD)					8.1–9.0
Total (without IBD)					7.7–8.0

a USA population-based cancer incidence data were obtained from registries that participate in the CDC's National Program of Cancer Registries and/or the NCI's Surveillance, Epidemiology, and End Results (SEER) Program.

b Cancer prevalence or incidence per 10,000 population was calculated with the assumption the population consists of 50/50 male-to-female.

c Incidence of autoimmune diseases reported for North America include Multiple Sclerosis, Type I Diabetes, Primary Biliary Cirrhosis, Autoimmune Hepatitis, Graves' Disease, Coeliac Disease, Addison's Disease, Sjogren's Syndrome, Systemic Lupus Erythematosus and Rheumatoid Arthritis. See Wang, L., Wang, F., and Gershwin, M.E. (2015). Human autoimmune diseases: a comprehensive update. Journal of Internal Medicine, Volume 278, Issue 4, Pages 369-395.[18]

7.9 Interfering Substances

There are no known interfering substances with the Cologuard Plus test. The Molecular and Hemoglobin assays of the test were challenged independently with the substances that could potentially be found in patient samples, including common lotions and creams, feminine over the counter products, stool softeners, anti-diarrhea products, laxatives, antacids, upset stomach relief products, anti-fungal medications, pain relievers, decongestants, stool softeners, urine, alcohol, Vitamin C, iron, common vegetables and fruits, fats, hypomethylation agents, and lipids. There was no observed interference with any substance in either assay. The hemoglobin assay was also tested with antibiotics, anti-inflammatories, anti-fungal drugs, pain relievers, and decongestants with no observed interference. The assays were tested with animal genomic DNA, hemoglobin, and/or myoglobin from commonly eaten animals with no observed interference.

8 Ordering the Cologuard Plus Test

The Cologuard Plus test is available for clinicians to order through Electronic Health Records (EHR) integrated ordering and resulting. Additional ordering options (e.g., EpicCare Link, efax and paper order forms) can be accessed from Exact Sciences Laboratories at http://www.cologuardplus.com.

The Cologuard Plus test includes a patient navigation program that provides attentive service to physicians and patients with live specialists. For any questions about the Cologuard Plus test or specific questions on how to order the test, please contact Exact Sciences Laboratories.

Exact Sciences Laboratories
145 E. Badger Rd, Suite 100
Madison, WI 53713
USA
844-870-8870

8.1 Sample Collection

- Samples for use with the Cologuard Plus test must be collected with the Cologuard Plus Collection Kit, including a stool sample for DNA testing (Container) and a stool sample for Hemoglobin testing (Tube and Probe).
- Patients should not provide a sample if they have diarrhea or blood in their urine or stool from bleeding hemorrhoids, bleeding cuts or wounds on their hands, rectal bleeding, or menstrual bleeding.
- Patients should familiarize themselves with detailed information contained in the Cologuard Plus Patient Guide and collection instructions before completing sample collection.
- The use of this kit requires sitting down on the toilet and standing up from the toilet. Patients should have someone available to help them sit down or stand up if needed.
- To ensure the integrity of the sample, the laboratory must receive patient specimens within 144 hours of collection. Patients should send stool samples to the laboratory using UPS™ according to the instructions included in the Cologuard Plus Collection Kit.

8.2 Instructions for Sample Collection

Once the Cologuard Plus test has been ordered, the patient will receive a Cologuard Plus Collection Kit. Detailed instructions for patient specimen collection are provided in the instructions accompanying the Cologuard Plus Collection Kit. Full closure of the stool collection container should be emphasized to patients to ensure receipt of a usable sample for testing. A toll-free number is also provided with the patient guide to ensure that any patient questions are addressed. An overview of the 7 steps in the collection process is provided in the following figure.

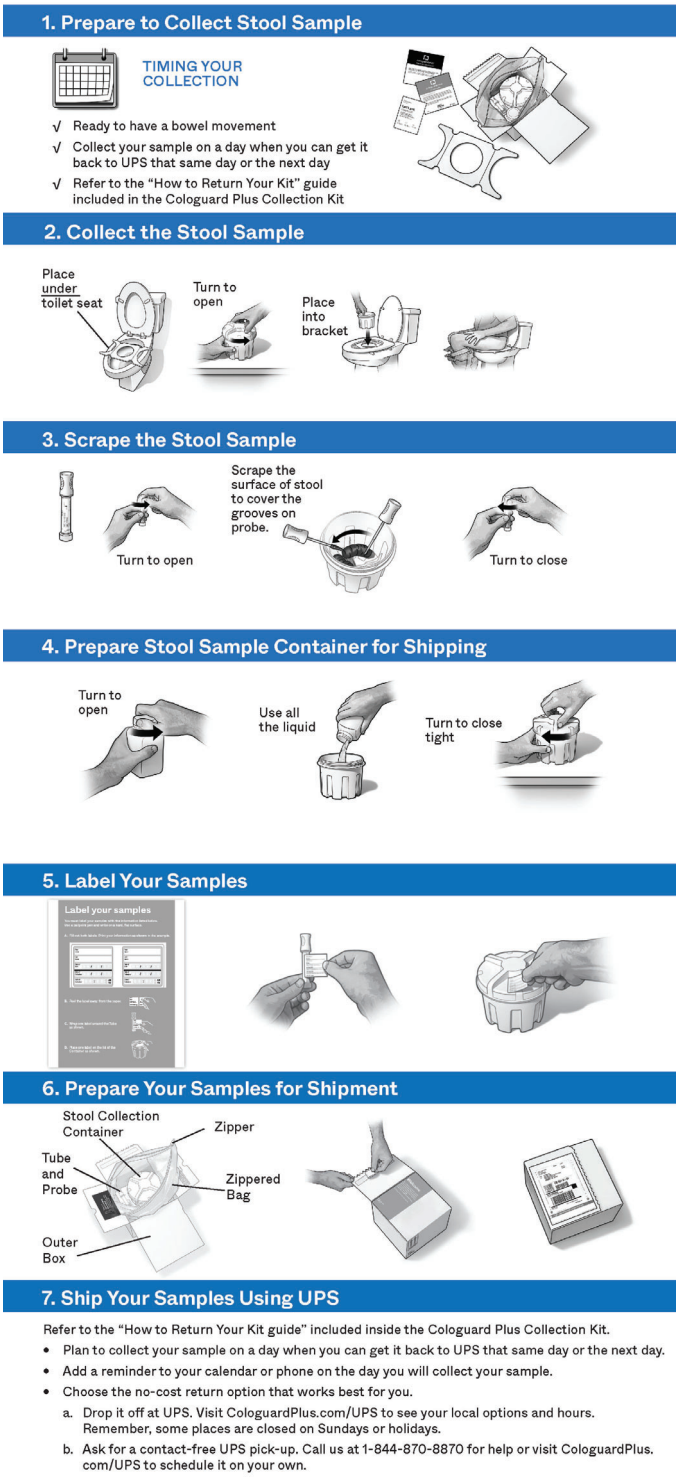


Figure 8-1: Collection Process

8.3 Interpretation of the Cologuard Plus Test Results

A negative test result means that the test did not detect signs of APL (precancer) or CRC in the stool sample. A test can also have a negative result that is

incorrect (false negative). For that reason, it is important to continue a regular screening schedule with your patients.

- Out of every 10,000 patients testing negative, approximately 2 will be falsely reassured that they do not have CRC. Out of every 100 patients testing negative, approximately 7 patients will be falsely reassured that they do not have APL.

A positive Cologuard Plus test result means that the test detected possible signs of precancer or CRC in the stool sample. A test can also have a positive test that is incorrect (false positive). Any positive result should be followed by a colonoscopy.

- Out of every 100 patients testing positive, approximately 3 patients will have CRC, 34 patients will have APL, 33 will have a non-advanced adenoma, and 30 will have no neoplastic findings.

In some cases, the Cologuard Plus test may not generate a result. If this occurs, a new patient sample may be requested.

9 References

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