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IVD For In Vitro Diagnostic Use

REF CRCDX-RAS

Σ 40 tests

CRCdx® RAS Mutation Detection Kit

Instructions for Use – Version 1.2

For use with

EntroGen PCR Analysis Software (EPAS™) - Version 2.0

For in vitro diagnostic use

For use with QuantStudio™ Dx real-time PCR instrument

For use with Promega Maxwell® Clinical CSC automated nucleic acid extraction system

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Contents

1. Intended Use

The CRCdx® RAS Mutation Detection Kit is a qualitative real-time PCR *in vitro* diagnostic test intended for the detection of 35 variants of KRAS and NRAS exon 2, 3, 4 somatic mutations in genomic DNA extracted from formalin-fixed, paraffin-embedded (FFPE) colorectal cancer (CRC) tissue samples. The test is intended as a companion diagnostic (CDx) to aid in the identification of colorectal cancer (CRC) patients who may benefit from treatment with Vectibix® (panitumumab) based on a no mutation detected test result in accordance with the approved therapeutic product labeling.

The CRCdx® RAS assay is performed on the QuantStudio™ Dx real-time PCR instrument (QSDx) for testing analyses and data collection. The data are analyzed by EntroGen's PCR Analysis Software (EPAS™) for result interpretation.

2. Product Description

2.1. Background

RAS family oncogenes encode GTPases that play a key role in transducing signals from the epidermal growth factor receptor (EGFR) to downstream effectors resulting in cell proliferation and differentiation. KRAS and NRAS mutations have been commonly found in several types of human malignancies, such as metastatic colorectal cancer (mCRC), lung adenocarcinoma and thyroid cancer. The most common mutations are found in KRAS codons 12, 13 and NRAS codon 61 [1, 2].

Several studies have demonstrated that tumors carrying any of these aberrations of the KRAS or NRAS genes are less likely to respond to anti-EGFR antibody therapy [3, 4, 5]. The American Society of Clinical Oncology (ASCO) recently released its first Provisional Clinical Opinion (PCO) suggesting that all patients to be administered anti-EGFR monoclonal antibody therapy should be screened for KRAS mutations [6, 7]. Additionally, the National Comprehensive Cancer Network (NCCN) has the following clinical practice guidelines: “Patients with any known KRAS Mutation (exon 2 or non-exon 2) or NRAS Mutation should not be treated with either cetuximab or panitumumab (Vectibix®)” [8, 9]. These findings strongly suggest that screening for both KRAS and NRAS mutations is necessary to more accurately identify patients who will not respond to anti-EGFR therapy.

2.2. Principle of the Procedure

Mutation detection is achieved by PCR amplification of target regions of the KRAS and NRAS genes using allele-specific oligonucleotide primers, fluorescent hydrolysis probes, and a hot-start DNA polymerase. Each reaction contains two sets of primers and probes, one set for amplification of target regions of the KRAS or NRAS genes with a FAM-labeled probe, and one set for amplification of an endogenous control gene (beta-2-microglobulin; B2M) with a CAL® Fluor Orange 560-labeled (CFO560) probe. B2M is an unrelated gene that acts as an internal control (IC); it is used to ensure that sufficient quality and amount of DNA is available for amplification. The IC is amplified in all samples, regardless of the presence of a mutation in the KRAS or NRAS genes.

2.3. Pre-analytical/Tumor Content Requirements

The EntroGen CRCdx® RAS Mutation Detection Kit requires that the formalin-fixed paraffin-embedded (FFPE) tumor sample must be confirmed to have at least *20% or greater tumor content*. Tumor content can be evaluated by having the tissue sectioned onto glass slides which then can be stained and examined for tumor content under a microscope by qualified pathology professionals.

2.4. Sample Preparation

The CRCdx® RAS Mutation Detection test kit does not contain reagents for extraction of genomic DNA from tumor samples. Genomic DNA from formalin-fixed paraffin-embedded (FFPE) tissues was validated using the Maxwell® CSC DNA FFPE Kit (Promega Cat # AS1350), with whom EntroGen has a supplier agreement. This assay requires the use of molecular grade water (nuclease free). It requires a total of 192 ng of DNA per sample (24 ng/reaction). If a single DNA extraction is performed (~45 µl remaining eluate), sample concentration should be 4.26 ng/µl or greater. DNA sample concentration should be at least 2.67 ng/µl if testing more than one pooled extraction.

Note: Quality of DNA isolated from FFPE blocks can vary due to fragmentation. Therefore, the DNA concentration does not reflect the amount of amplifiable DNA available for the assay. When targeting a VIC Ct, one may need to load more or less than 24 ng of sample.

2.5. Target Mutations

Table 1. List of KRAS and NRAS Mutation Detected by CRCdx® RAS

Gene	Exon	AA change	NT change	Cosmic ID (COSV)	Detected by Primer No.	CRCdx® Reaction Name
KRAS	2	G12A	c.35G>C	55497479	1	KRAS 12/13
		G12D	c.35G>A	55497369		
		G12R	c.34G>C	55497582		
		G12V	c.35G>T	55497419		
		G13D	c.38G>A	55497388		
	2	G12C	c.34G>T	55497469	2	KRAS 12
		G12S	c.34G>A	55497461		
	3	Q61H	c.183A>T	55499223	3	KRAS 59/61
		Q61H	c.183A>C	55498802		
		Q61L	c.182A>T	55504296		
		Q61R	c.182A>G	55498739		
		A59E	c.176C>A	55568979		
		A59G	c.176C>G	55604554		
		A59T	c.175G>A	55499283		
	4	K117N(AAC)	c.351A>C	55545304	4	KRAS 117
		K117N(AAT)	c.351A>T	55504752		
		K117R	c.350A>G	55937655		
		K117E	c.349A>G	55716212		
		A146T	c.436G>A	55501778	5	KRAS 146
		A146P	c.436G>C	55541748		
		A146V	c.437C>T	55498939		
NRAS	2	G12D	c.35G>A	54736383	6	NRAS 12/13/117
		G12C	c.34G>T	54736487		
		G12S	c.34G>A	54736621		
		G13R	c.37G>C	54736550		
		G13V	c.38G>T	54736480		
	4	K117R	c.350A>G	104682079		
	3	Q61H (CAC)	c.183A>C	54736320	7	NRAS 61
		Q61H (CAT)	c.183A>T	54736991		
		Q61L	c.182A>T	54736624		
		Q61K	c.181C>A	54736310		
		Q61R	c.182A>G	54736340		
	3 and 4	A146T	c.436G>A	65731978	8	NRAS 59/146
		A59D	c.176C>A	54738004		
		A59T	c.175G>A	54743124		

2.6. EPAS™ - Automated Mutation Results Interpretation

Upon run completion, the QSDx software[1] (OTS software included with QSDx) uses the predefined analysis parameters (i.e., threshold and baseline settings) to generate Ct (cycle threshold) values for each well and records the values in a data file (EDS file). The Ct values represent the cycle at which the fluorescence crosses the predefined threshold. The EDS file is then imported into the EntroGen PCR Application Software (EPAS™), a proprietary software developed by EntroGen and installed on a stand-alone workstation PC, to determine sample's mutation status based on the Ct values of the IC and target for all 8 CRCdx® reactions.

EPAS™ analyzes Ct values of two different reporter dyes, VIC (Internal Control) and FAM (mutation target) values based on the assay/instrument parameters to determine the output results of the NTC (non-template control/negative control), the positive control (PC) and the tested samples. EPAS™ is configured with specific acceptance ranges for both the IC and mutation targets. The validity of each run is measured by the included Positive Control (PC) and No Template Control (NTC). EPAS™ presents the analyzed results as PASS/FAIL for the Controls, PASS/FAIL for IC for each sample and reaction group name (positive)/negative. Since the assay is multiplexed, some loci-specific mutations will be indistinguishable with each 'mutant' call – see Table 1 above.

[1]*ThermoFisher qPCR Instrumentation*

3. Precautions and Handling Requirements

3.1. Warnings and Precautions

This test is to be used for CRC FFPE samples. All samples should be handled as if infectious using safe laboratory procedures such as those outlined in CDC's Biosafety in Microbiological and Biomedical Laboratories and in the CLSI Document M29-A4 [10, 11]. Clinical laboratory practices pursuant to Good Clinical Laboratory Practice (GCLP) is recommended as applicable [12].

3.2. Assay Limitations

- For prescription use only. This test must be ordered by a qualified medical professional in accordance with clinical laboratory regulations.
- Successful detection of target listed KRAS and NRAS mutations is dependent on the overall aspects of percent tumor minimum requirement (sufficient genomic DNA to be detected), adequate sample integrity, and absence of interferences and/or inhibitors.
- The performance of this assay has not been established in the presence of rare polymorphisms.
- The use of a spectrophotometer for sample quantification may under- or over-estimate concentration. The input recommendations in this assay are based on fluorometric analysis. The performance of this assay has not been established with spectrophotometric quantification and the input may require adjustment.
- FFPE fixation may over-fragment samples. Excessive fragmentation may lead to assay failure. As a result, some samples may not be reportable.
- A negative result does not rule out the presence of a mutation below the limits of detection of the assay.
- This assay is intended to be used by laboratory personnel trained in molecular biology techniques.
- EPAS™ for CRCdx® reports the presence of a single mutation. Additional KRAS or NRAS mutations may be present.
- Combination of reagents from different kit lots has not been evaluated. The performance characteristics of this modification have not been established.

3.3. Quality Practice Guidelines

- Do not use kits after their expiration dates.
- Do not mix or pool reagents from different kits or lots.
- All disposable items are for one time use. Do not reuse.
- All equipment should be properly maintained according to the manufacturer's instructions.
- Appropriately trained and organized laboratory personnel are key to the successful operation.
- Individual assay controls must be in place to ensure assay performance, i.e., for qualitative tests, include positive and negative controls with each run.
- Molecular amplification procedures within the laboratory that are not contained in closed systems must have a unidirectional workflow. This must include separate areas for specimen preparation, amplification, detection, and as applicable, reagent preparation to avoid contamination and mix-ups between test and control articles.

3.4. Cross Contamination Precaution

PCR amplification is extremely sensitive to cross-over contamination. The use of sterile disposable pipettes and DNase-free pipettor tips is recommended. It is very important and highly recommended to carry out the pre-amplification steps (i.e., DNA isolation, PCR reaction preparation) in separate areas and conduct workflow in a unidirectional manner by completing one activity before proceeding to another activity, ideally in separate rooms with isolated air venting systems. It is recommended that the PCR reaction preparation is carried out in a room with positive air pressure (or laminar flow hood).

Additional precautions are necessary when handling DNA and PCR reagents to avoid contamination between samples/reagents:

- Wipe down all work areas, pipettes, and equipment to be used near the work area with surface decontaminant (20% bleach or equivalent) to eliminate DNA and DNase followed by 70% ethanol.
- For personal protection, wear eye goggle, laboratory coats and disposable gloves when handling any reagents. Avoid contact of these materials with the skin, eyes or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills occur, dilute with water before wiping dry. Seek emergency procedure when necessary.
- Gloves must be changed frequently to reduce the potential for contamination.
- Gloves must be changed after DNA extraction or if contact with solutions or a sample is suspected.
- Avoid microbial contamination of reagents.
- The amplification and detection work area should be thoroughly cleaned before proceeding to other preparation. Supplies and equipment should be dedicated to each activity and not used for other activities or moved between areas. For example, pipettors and supplies used for DNA extraction must not be used to prepare reagents for amplification and detection.
- Vortex each tube before use.
- Spin down all tubes in picofuge before opening.
- Open each microcentrifuge tube carefully after vortexing/mixing and avoid touching the inside of the lid as there may be drops of liquid inside the lid.
- Use aerosol-resistant (filtered) tips for all pipetting steps to avoid cross-contamination.
- Change pipette tips between all liquid transfers.
- Always wear gloves when handling DNA/reagents and change gloves between pre-amplification and post-amplification steps.
- The flow of tubes, racks, pipettes and other equipment used should be from pre-amplification to post-amplification, and never backwards.
- *Do not pipette by mouth*.
- Do not eat, drink or smoke in laboratory work areas.
- Wash hands thoroughly after handling samples and kit reagents.

4. Reagents and Instruments

4.1. Package Contents

This product contains the following materials sufficient for up to 40 patient specimen reactions per kit:

Table 2. CRCdx® RAS Mutation Kit Reagents

Name	No. of Tubes	Vol. per Tube (µl)
Mutation Detection Reaction Mix (2X)	5	1300
RAS Primer Mix (1-8)	8 [3]	300
RAS Positive Control Mix[2]	2	200

[2]The positive control mix contains a mixture of synthetic DNA sequences that correspond to a representative mutation per reaction detected by this kit in a background of wild-type genomic DNA.

[3]Each tube contains a distinct primer/probe mix to detect its designated RAS mutations. The contents of these tubes should not be mixed.

4.2. Handling and Storage

- This product is shipped on frozen ice packs (-25^0 to -15^0 C). Inspect content upon receipt to ensure reagents are received frozen. Please contact EntroGen tech support if reagents were completely thawed at the time of receipt.
- Shipments contain temperature monitoring devices that change color based on exposure to >8°C brief (2 hrs), moderate (12 hrs), and prolonged (48 hrs). The device should indicate no temperature excursions. Please contact EntroGen tech support if a temperature excursion was detected prior to use of the kit.
- The contents of the shipment should be stored between *-25°C and -15°C* in a non-frost-free freezer immediately upon receipt.
- Store all unopened components in original containers.
- Primer/probe mixes should be protected from light at all times to prevent photo bleaching of the fluorescent dyes. Perform assay setup with the hood light turned off, as probes can be photo-bleached once aliquoted into the reaction plate from direct light exposure from the hood light.
- Centrifuge the tubes before opening.
- The expiration date of each component is printed on each tube label. This product will maintain its performance through this date. Its performance is not guaranteed if used after the expiration date with the environment storage indicated.

4.3. Reaction Master Mix

Store frozen between -25°C to -15°C. After each use, Reaction Master Mix should be stored at -25°C to -15°C. If the Reaction Master Mix is expected to be fully consumed quickly, storage at 2°C to 8°C is acceptable for no more than 4 weeks. Do not freeze-thaw this reagent more than 5 times, as this repeated freeze-thaw >5 times, will be out of scope and adversely affect the efficiency of the enzyme. If a single vial of this reagent is expected to be used longer than 4 weeks, small volume aliquots are recommended after the first thaw.

4.4. Primer/Probe Mix

Store frozen between -25°C to -15°C. After each use, primer/probe should be stored at -25°C to -15°C. If the primer/probe is expected to be fully consumed quickly, storage at 2°C to 8°C is acceptable for no more than 4 weeks. Do not store primer/probe mixes at 2°C to 8°C for longer than a total of 4 weeks. Avoid repeat freeze-thaw cycles (more than 5) as it will adversely affect the results.

4.5. Positive Control Mix

Store frozen between -25°C to -15°C. After each use, the Positive Control Mix should be stored at -25°C to -15°C. If the Positive Control Mix is expected to be fully consumed quickly, storage at 2°C to 8°C is acceptable for no more than 4 weeks. Do not store Positive Control Mix at 2°C to 8°C for longer than a total of 4 weeks. Avoid repeat freeze-thaw cycles (more than 5) as it will adversely affect the results.

4.6. Pre-analytical Procedure Reagents

Procedural master mixes (Reaction Master Mix + Primer/Probe Mix + Water) can be stored in the dark at *2°C to 8°C* or 18*°C to 26°C* for up to 2 hours after mixing. Assembled plates can be stored in the dark at *2°C to 8°C* or 18*°C to 26°C* for up to 6 hours. Assembled plates should always be centrifuged immediately preceding loading into the instrument. Storage and handling of the components of this product under conditions other than those described above may affect the performance of this assay and adversely affect the results

4.7. Quality Control

The components of this product are manufactured under cGMP (current Good Manufacturing Practice) regulations. cGMP requires extensive validation and documentation of manufacturing procedures to ensure highest production quality.

Each batch is tested on the QuantStudio™ Dx instrument. Lot-specific Certification of Analyses are available upon request or for download at entrogen.com/pi-downloads.

4.8. Workflow Guideline for CRCdx® RAS Testing Analysis

Table 3. Workflow Guideline for CRCdx® RAS Testing Analysis[4]

Step	Description	QC Check
1	Start instrumentation	The Instrumentation performs a self-check to ensure proper function
2	Perform or check necessary calibration	An automated notification will alert the user when a calibration is out of spec and the run will not proceed.
3	Assemble Plate for Analysis	Visual check to ensure proper plate sealing and appropriate volumes are in the wells.
4	Run QuantStudio™ Dx protocol	The .tdd file provides the instrument with the sample layout, thermal cycling parameters, and analysis workflow. An automated notification will alert the user of any instrumentation mechanical errors.
5	Analyze .eds data file using EPAS™	Results are validated using a positive control, a non-template control, and an internal control. Ct results are evaluated by EPAS™ and the results report will alert the user if the run/sample data is acceptable or not acceptable.

[4]per recommended PCR procedure & environment. See sec. 3.4.

5. Required Equipment & Materials

5.1. Samples and DNA Genomic Materials

Only CRC FFPE samples containing a minimum of 20% tumor shall be used for this test. Eight (8) 5-µm FFPE curls is required per extraction. A total of 192 ng of DNA per sample (24 ng/reaction) is recommended for an individual test, however additional testing may be required depending on the quality of the sample. Promega Maxwell® CSC DNA FFPE Kit (Cat # 1350) has been validated to extract DNA genomic materials for CRCdx® RAS Mutation Detection Kit.

5.2. Reagents

This kit does not contain reagents for extraction of genomic DNA from tumor samples. Genomic DNA from formalin-fixed paraffin-embedded (FFPE) tissues should be extracted using Maxwell® CSC DNA FFPE Kit extraction method (Promega Cat # AS1350).

This assay requires the use of molecular grade water (nuclease free) - not provided.

5.3. Equipment and Materials

Equipment and materials required (not provided) to perform this assay are:

- Applied Biosystems QuantStudio™ Dx Real-Time PCR instrument
- Disposable powder-free gloves
- Adjustable pipettes
- Sterile filtered pipette tips
- Vortex mixer
- Microcentrifuge
- Centrifuge for PCR plates
- 96-well Semi-Skirt 0.1mL PCR Plate
- Optical Adhesive Film
- 2.0 ml microcentrifuge tubes
- Molecular Grade Water
- 70% ethanol
- 20% chlorine bleach (or equivalent)

6. Instructions for Use

6.1. DNA Isolation

- The Promega Maxwell® CSC FFPE kit (Cat # AS1350) has been validated to isolate DNA from FFPE tissue for CRCdx® testing analysis and to support the assay performances.
- Follow the genomic DNA isolation procedure according to manufacturer's protocol to extract DNA from eight (8) 5-µm curls per extraction tube.
- Measure the DNA concentration using a Qubit® Fluorometer. DNA concentration from a single extraction should be 4.26 ng/ul or greater to reach the required target input. Lower DNA yields require additional extractions or curls with greater biomass content.
- The recommended DNA input for the CRCdx® RAS Mutation Detection Kit is 24 ng per reaction or 192 ng per sample.
- CRCdx® evaluates DNA input concentration using an internal control reaction (VIC) Ct to indicate a sufficient load. A sample load adjustment may be required because the quality of DNA isolated from FFPE blocks may vary due to fragmentation.

6.2. Reagent Preparation

- Thaw all of the primer/probe mixes, positive control mix, and 2X reaction master mix (Cat # BLSM2).
- Vortex the tubes well to assure no frozen components exist (5-10 secs).
- Spin the tubes briefly at room temperature to collect the contents at the bottom of the tubes.
- The PCR reactions are setup in a total volume of 30 µl/sample.
- Reaction mixes for multiple samples (as well as control samples) should be pre-mixed as a master mix with 5% overage to cover pipetting errors.
- 3µl of the positive control is tested with each reaction on every plate.
- 9µl of mol. grade water (not included) for NTC is tested with each reaction on every plate.

The following volumes go into each 30 µl reaction:

Table 4. CRCdx® Reaction Composition

Components	Volume
2X mutation detection reaction mix	15 µl
Primer mix (1-8)	6 µl
DNA sample or PC	Up to 9 µl sample (3 µl if PC)
Molecular grade water	adjusted to 30 µl total reaction volume

6.3. Plate Setup

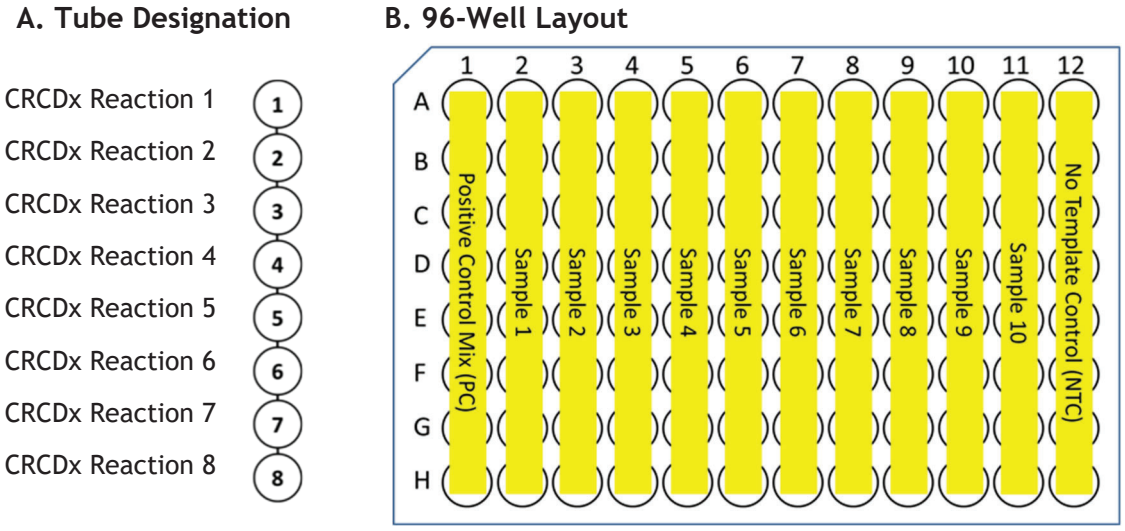
Eight reactions are setup for each sample. A single 96-well PCR plate can accommodate up to 10 samples, one positive control mix, and one no template control as seen in Figure 1 below.

1. Prepare a master mix for KRAS and NRAS detection for each CRCdx® primer mix by mixing 2X PCR reaction mix, primer mix, and molecular grade water with 5% overage.
2. Mix by vortexing and briefly centrifuge at room temperature.

3. Dispense the master mix per well into a single row (i.e., each row on the 96-well plate (USA Scientific Cat # 1402-9100) represents a single primer mix reaction). See Figure 1 A. Tube Designation layout below.
4. Add 24 ng of DNA (up to 9 µl of sample) or 3 µl of PC to its corresponding well according to the layout shown in Figure 1 B. 96-Well Layout below.
5. Seal the plate with the optical sealing film.
6. Briefly centrifuge the plate to collect reaction at the bottom of the wells and to ensure that there are no bubbles in the bottom of the wells.

6.4. Plating Map Example

Figure 1. CRCdx® Layout for a Single Test Plate Analyzing 10 Unknown Samples



6.5. Instrument Setup

Applied Biosystems QuantStudio™ Dx (in IVD mode):

1. Turn on the QuantStudio Dx in IVD mode.
2. Open the QuantStudio Dx software in IVD mode.
    a. Enter the username and password
3. Highlight “CRCDx” under TDD name, and select “Setup” (refer to Section 7.2 for directions on how to install a .tdd file)
4. Under Setup → Test Properties, give the run a unique identifier name.
5. Under Setup → Define, insert the sample names.
    a. Positive control must be labeled as “PC” and No Template Control must be labeled as “NTC” in order for the software to analyze the data correctly.
    b. Each sample column must have a unique name assigned to it in order for the run to initiate. The QSDx-IVD software will prompt an error at the ‘Start Run” phase if the default samples are not changed.

   c. Label empty or blank columns that do not contain any sample, PC, or NTC as successive "Blank" (i.e., Blank1, Blank2, Blank3, etc.)
6. The well positions are automatically assigned based on the order entered on the define tab.
7. View the plate layout under Setup → Assign.
   a. Ensure all columns containing sample are labeled correctly and appear in the correct column as loaded in the plate (refer to Figure 1 for example layout).
   b. Highlight each empty/blank column individually and uncheck the columns in the "Samples" dialog box, located on the left side of the screen.
   c. ***Warning*** *– failure to uncheck blank/empty columns will cause the QSDx-IVD software to report abnormal Ct values. When the samples names from empty/blank wells are deselected, EPAS™ will not interpret data produced for those wells.*
8. On the touch screen on the instrument hit the red eject button to open the tray. Once the plate is loaded, hit the eject button again to retract the try inside the instrument.
   a. Make sure well A1 (notched) is in the upper left-hand corner.
9. In the computer software, select Run, press the green "Start Run" button and select the serial number of the instrument to be used.

7. Instrumentation

7.1. QuantStudio Dx Real-Time PCR Instrument

The QuantStudio Dx real-time PCR instrument is a diagnostic device developed, manufactured, and distributed by ThermoFisher® Scientific. It comes with a 96-well block that enables testing of up to 10 samples per run with the CRCdx® RAS assay. The instrument includes a touch-screen and a desktop computer that runs the QuantStudio Dx software.

The QuantStudio Dx real-time PCR instrument is controlled by the QuantStudio Dx software which uses a test definition document (TDD), a locked file with predefined run and analysis parameters, to control the IVD test and must be locally installed prior to running CRCdx® RAS (See Section 7.2). The QuantStudio Dx software maintains secure access to the instrument, enables assignment of specific software function privileges of users, records all actions completed by users, and signs data electronically. The instrument, its on-board software engine, and the QuantStudio Dx user interface software were developed under design control process and related software standards by ThermoFisher® Scientific and is compliant with 21CFR Part 11.

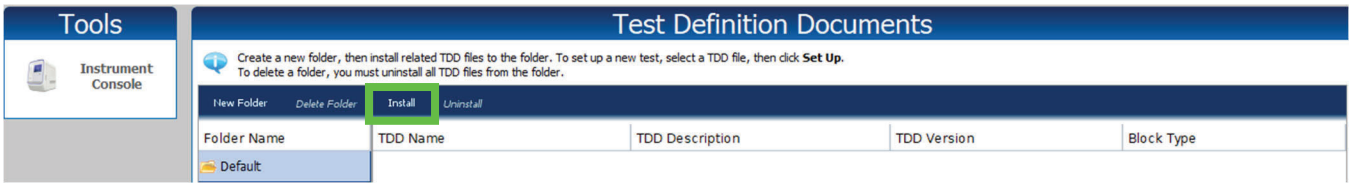
7.2. Test Definition Document (TDD)

Please follow the instructions below to install the TDD file on to the QuantStudio Dx prior to using CRCdx®.

Install the CRCDx_v1_5_Certified_IVD.tdd file in the Quantstudio Dx Software

1. Turn on the Quantstudio Dx in IVD mode.
2. Open the Quantstudio Dx Software v.1.0.3 in IVD mode.
3. Click Install (green box) on the Test Menu

Figure 2. QSDx Software screenshot for uploading TDD file



4. Open the CRCDx_v1_5_Certified_IVD.tdd provided by EntroGen.
5. CRCDx version 1.5 will now appear as a test in the setup menu.
6. Create a folder in My Documents with the following path:

D:\Users\INSTR-ADMIN\Documents\EntroGen_Experiments

All data will automatically be exported to this folder.

8. EntroGen PCR Analysis Software (EPAS™)

8.1. EPAS™ Description

EPAS™ (EntroGen PCR Analysis Software) is a custom software developed by EntroGen intended to be used as a tool to aid in the interpretation and reporting of CRCdx® RAS Mutation Detection real-time PCR results.

EPAS™ functions independently and has no involvement with the host device functionality. It does not support, supplement, and/or augment the performance of any devices, including the data generated by the ThermoFisher QuantStudio Dx™ (QSDx). The QSDx system runs and analyzes the results generated by the CRCdx® RAS Mutation Detection Kit according to parameters defined in the test definition file (TDD). TDD files are locked files that contain non-editable cycling conditions and Ct threshold settings. TDD files are created and supplied by EntroGen and certified by ThermoFisher. Upon completion of a CRCdx® run, the QSDx software saves the analyzed CRCdx® data file (EDS) in a user-specified directory on the QSDx system.

EPAS™ is a stand-alone application intended to be installed on a Windows®-based personal computing workstation. EPAS™ must be installed on an independent computer system (separate from the QSDx system) as a stand-alone application for data interpretation and reporting. The user utilizes EPAS™ to securely import the CRCdx® data file (generated by the QSDx system), enter traceability parameters (such as lot number, instrument ID, and performer), execute and display results, and generate a test report. The EPAS™ generated test reports are machine readable text files that are suitable for integration with laboratory information management systems (LIS/LIMS) for more streamlined reporting processes.

EPAS™ automates result interpretation and reporting by checking raw fluorescence values to ensure sample and reagents are properly loaded into each well and by comparing Ct values from the CRCdx® data file to Ct value ranges defined in the assay configuration file. EPAS™ utilizes a combination of these processes to designate PASS/FAIL results for both run control and sample quality metrics, and to determine the RAS mutation status for each sample tested in the analyzed data file. The EPAS™ application is compliant with 21CFR Part 11 and also provides a user-friendly system with system error alerts and system message prompts that serve to prevent erroneous test reporting for heightened patient security, safety, and protection.

8.2. Intended Use

EPAS™ is intended to be used as an accessory to the CRCdx® RAS Mutation Detection Kit when installed on a Windows®-based personal computing system and used as a tool to aid interpretation and reporting of real-time PCR results generated by the CRCdx® RAS assay. EPAS™ is intended to be used for In Vitro Diagnostic (IVD) purposes by trained laboratory technologists in combination with the CRCdx® RAS Mutation Detection Kit.

8.3. EPAS™ Functional Modules / Pages and Tabs

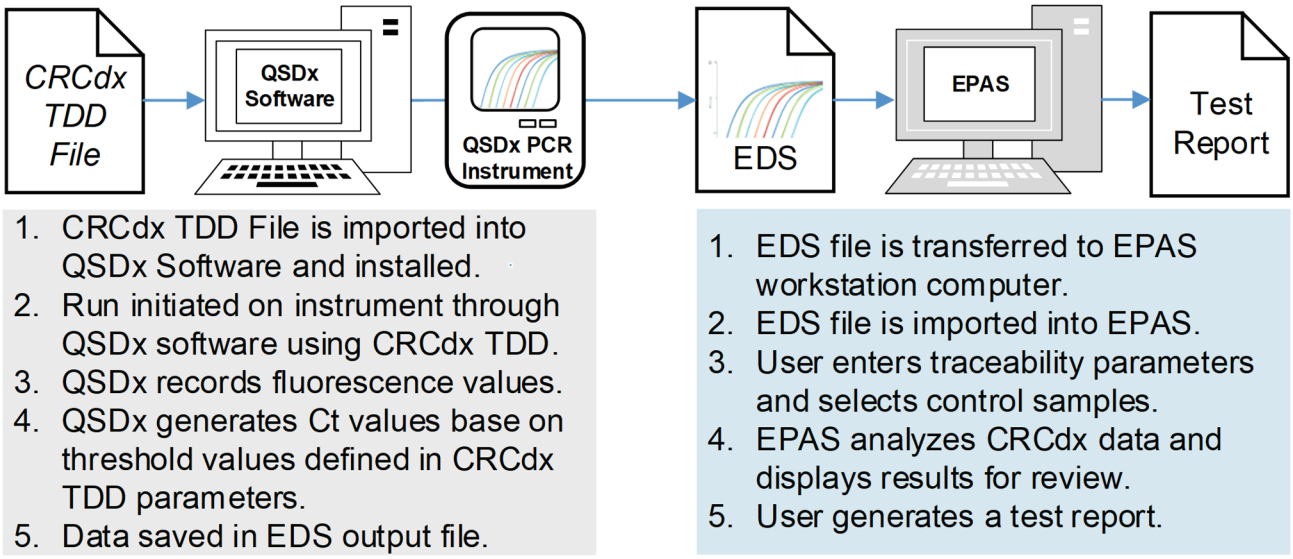
EPAS™ is comprised of the following **four main modules / pages** and their **sub-modules, sub-pages, or tabs**:

1. **Installation Module**: This module provides the necessary steps and tools for installing EPAS™ on a stand-alone workstation personal computer.
2. **Login Page**: This module provides the necessary authentication, authorization, and traceability mechanisms for authorized users to securely access EPAS™.
3. **Results Page**: This module provides the necessary tools and mechanisms for importing data, entering traceability and analytical parameters, and analyzing CRCdx® data files.
    a. **Data Imports Page:** This sub-module provides the necessary mechanisms for importing CRCdx data files into the EPAS™ application.
    b. **Parameters Page:** This sub-module is where assay traceability parameters (such as instrument identification IDs, CRCdx assay lot numbers, and personnel performing analysis) are entered and controls samples (such as positive and negative controls) are selected.
    c. **Analysis Page:** This sub-module is where the analytical results for the CRCdx® data file are presented for review. This sub-module also allows for run / sample annotation and test report generation.
4. **Administration Page**: This module provides the necessary tools for reviewing user activities and managing user accounts.
    a. **Activities Tab:** This sub-module is where user activities can be reviewed and exported by personnel with administrative or supervisory access privileges.
    b. **Users Management Tab:** This sub-module is where users with administrative access privileges can manage user profiles (such as create new users, edit existing users, and delete old users).

8.4. CRCdx® Assay Workflow on EPAS™

1. A CRCdx® run can only be started from an installed TDD file on the QSDx instrument.
2. The QSDx instrument records raw fluorescence data from the CRCdx® plate and determines Ct values based on the threshold settings locked in the TDD file.
3. The CRCdx® data file is transferred to the EPAS™ workstation computer in accordance with the laboratory's / institution's cybersecurity protocols.
4. The CRCdx® data file is securely imported into EPAS™.
    a. Note: EPAS™ will only accept and analyze CRCdx® data files produced in the IVD mode of the QSDx instrument. Data files produced from runs in RUO mode on the QSDx instrument are prevented from being imported or analyzed by EPAS™.
5. The user enters traceability parameters (such as lot number, instrument ID, and performer) and selects Positive and Negative Control Samples.
6. CRCdx® results are analyzed using EPAS™ and a test report containing the RAS mutation status is generated.

Figure 2. QSDx EDS file to EPAS™ Workflow Overview



Note: Control Ct values must fall within a pre-determined Ct ranges to be considered valid.

- TDD files are created and supplied by EntroGen. TDD files are locked files that contain non-editable cycling conditions and Ct threshold settings.
- EPAS™ analyzes the CRCdx® results according to the parameters defined in the TDD file by comparing the Ct values and raw fluorescence signals from the imported .eds file to acceptance criteria and mutation specific analytical cut-off values defined in the assay configuration file.

8.5. Software Limitations

1. All EPAS™ application windows, prompts, alerts, and pages are in English.
2. The EPAS™ software is intended for use by trained laboratory technologists and does **NOT** replace thorough evaluation of the data, including amplification plots, by personnel trained in molecular biology techniques.
3. Irregular readings of test wells due to instrument malfunction, user error (such as reagent omission), or other interferences / abnormalities may affect results. EPAS™ does not distinguish between true amplification and possible artifacts (for example, early Ct due to a bubble in the well or an elevated baseline).
4. EPAS™ has not been validated when installed on the QSDx computer and must be installed on a stand-alone workstation personal computer.
5. The workstation computer user must have administrative rights to install the EPAS™ application.
6. Multiple instances of EPAS™ cannot be run simultaneously and will cause an error message to appear. Similarly, multiple users on the same computer cannot be logged into an active session of EPAS™ simultaneously.
7. EPAS should be restarted regularly to maintain program integrity. Restart the program after analyzing several CRCdx data files in one session.
8. For added cybersecurity, EPAS™ will terminate any current session after 15 minutes of inactivity. A 5-minute warning message will appear after 10 minutes of inactivity asking if the

current session should remain active. Failure to reply Yes to the warning message will cause EPAS™ to logout after 5 minutes.

9. EPAS™ will only report the most prominent RAS mutation detected, specimens containing multiple RAS mutation will be reported under the mutation grouping with the lowest Ct value.

8.6. Operating System and Hardware Requirements

EPAS™ must NOT be installed on the QSDx computer and must be installed on a stand-alone workstation personal computer with the following software and hardware specifications:

8.7. Operating System Requirements

- Microsoft Windows 10 Home / Pro 64-bit operating system.

8.8. Hardware System Requirements

- Monitor resolution: 1280 x 1024-pixel monitor minimum
- Mouse: Scroll wheel mouse with 2 or more buttons
- Keyboard: MS compatible keyboard
- Minimum CPU or processor speed: Intel or AMD microprocessor with minimum 1.5GHz power (x86/x86-64 based chipset)
- System memory (RAM): 4GB RAM
- Computer port: 1 available USB-B 2.0 port minimum
- Energy sources: Standard 110-120V, 60Hz electric outlet to power the workstation computer.

EntroGen recommends that the stand-alone workstation personal computer running EPAS™ be equipped with anti-malware software. EPAS™ has been tested and is compatible with the following anti-malware applications:

- Microsoft Security / Windows Defender version 1.397.492.0
- McAfee AntiVirus version 16.0.R52
- Norton AntiVirus version 22.23.6.5

For more information about EPAS™ anti-malware compatibility please see the following for reference: <<<http://www.entrogen.com/crcdx-epas>>>

Users should perform a scan of the QSDx computer and any USB/flash drives received from EntroGen prior to installation of any software updates for the presence of viruses and malware to prevent introduction of viruses and malware that may adversely affect the test's performance.

8.9. EPAS™ User Account Requirements and Characteristics

8.9.1. User Account Requirements

- A valid EPAS™ Username must have 5 to 16 alphanumeric characters (underscores and dashes are allowed, but other punctuation marks are not).

- A valid EPAS™ Password must have 8 to 32 alphanumeric characters, with at least one uppercase character, one lowercase character, and one numeric character.
- All user accounts must have a Role Designation (see Section 8.9.2)

Note: EPAS™ will not allow for the creation of a user account that does not meet the username / password criteria stated above.

8.9.2. User Account Characteristics / Role Designations

EPAS™ has three (3) functional account level access privileges:

Administrator (admin):

- Has access to all EPAS™ features and system functions.
- Can access the Administration Page and all associated tabs: Activity Tab and Users Management Tab.
- Only admin level users are allowed to:
    - Delete imported data files from the Data Imports Page.
    - Create, edit, and delete user accounts.

Supervisor:

- Has access to all Results Page features: Data Imports, Parameters, and Analysis pages.
    - Can only import .eds data files
    - Cannot delete imported data files.
- Can access the Administration Page, but can only access the Activity Tab to review and export user activity logs.
    - Cannot create, edit, or delete user accounts.

Standard User (user):

- Has access to all Results Page features: Data Imports, Parameters, and Analysis pages.
    - Can only import .eds data files
    - Cannot delete imported data files.
- Cannot access the Administration Page.
    - Cannot review or export user activity logs
    - Cannot create, edit, or delete user accounts.

User accounts can be selected to be active or inactive by the administrator. Active accounts can login to EPAS™ freely and have access privileges appropriate for their role designation. Inactive accounts cannot access EPAS™ until activated by an account with administrative privileges.

Table 5. Summary of EPAS™ Role Designation Access Privileges

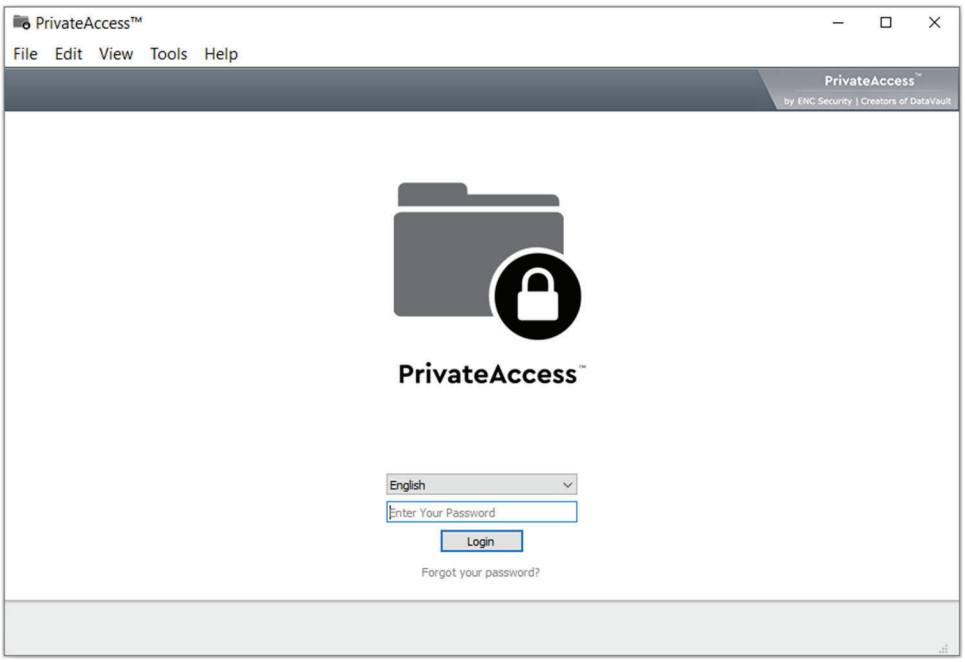
	Role		
Action	**Administrator (Admin)**	**Supervisor**	**User**
Login	• Logged out of EPAS™ after 15 minutes of inactivity		
Monitor Activities	• View and Export activity logs	• View and Export activity logs	• No Access
Manage User Roles	• Create user accounts • Assign role access privileges • Setup passwords • Reset passwords • Modify usernames • Activate / inactivate user accounts • Delete user accounts	• No Access	• No Access
Manage Results Database	• Import .eds data files • Delete imported data files	• Can only import .eds data files	• Can only import .eds data files
	• Add notes to sample comments and run comments fields		
Input Parameters	• Log instrument, reagent, and performer information (traceability parameters) • Assign positive and negative control samples • Analyze results		
Analysis	• View all sample results and assess run / sample quality metrics		
Reporting	• Export analysis reports		

9. EPAS™ Installation

The EPAS™ installation package contains all necessary components required to run EPAS™ on the stand-alone workstation computer. The Windows® Computer user must have administrative rights to install the EPAS™ application. The EPAS installer executable file is stored on the EntroGen supplied USB 2.0 external drive under the PrivateAccess® Software.

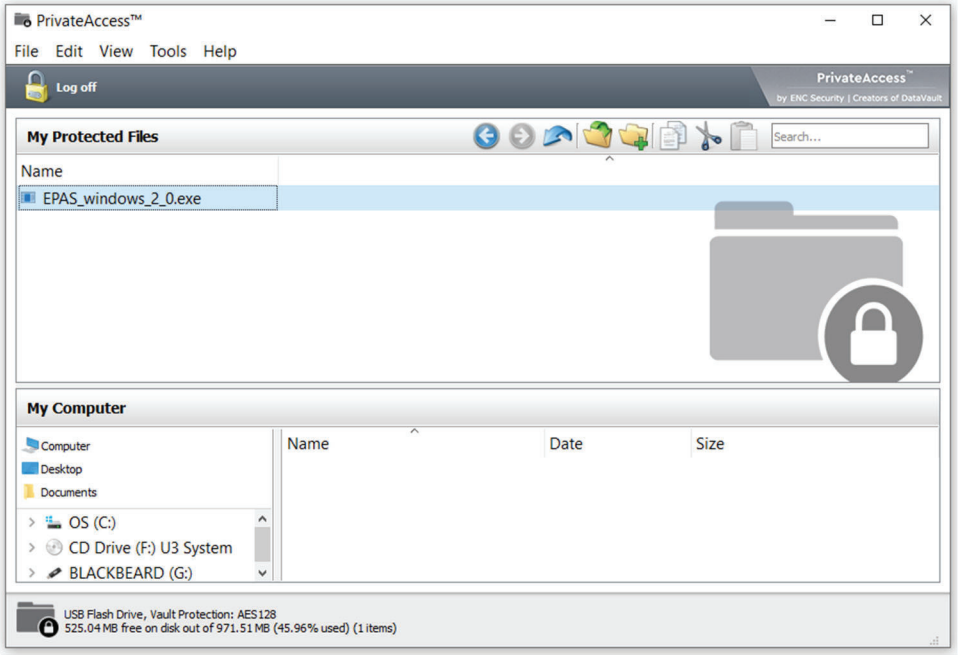
1. Insert the EntroGen supplied USB 2.0 external drive into the stand-alone workstation PC's USB port.
2. Navigate to the USB Drive root folder using Windows Explorer.
3. Double-click on the privateaccess-win.exe software to open.
4. Ensure the PrivateAccess language is set to English and enter the EntroGen supplied USB password.

Figure 3. PrivateAccess Login



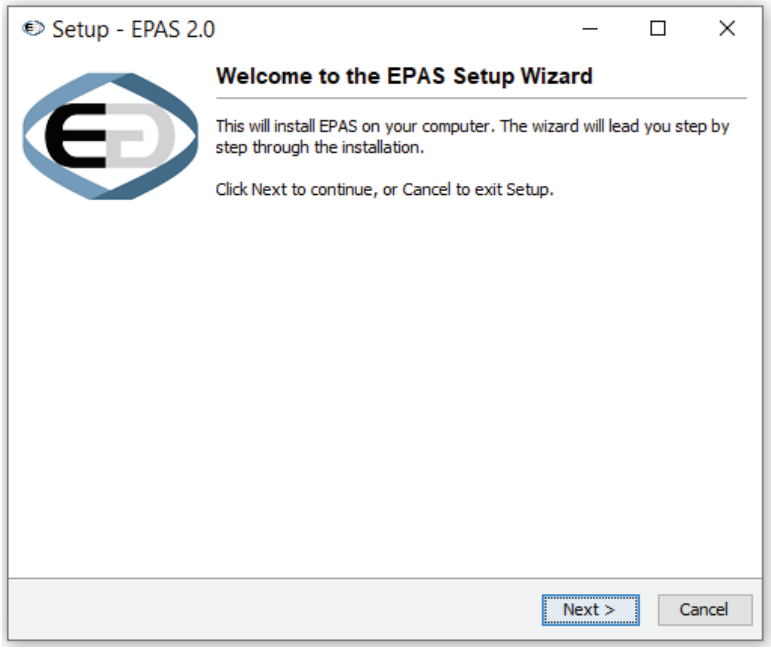
5. Click the **Login** button.
6. Double-click on the EPAS_windows_2_0.exe file to initiate EPAS™ installation on the PC.

Figure 4. EPAS Installer Executable File



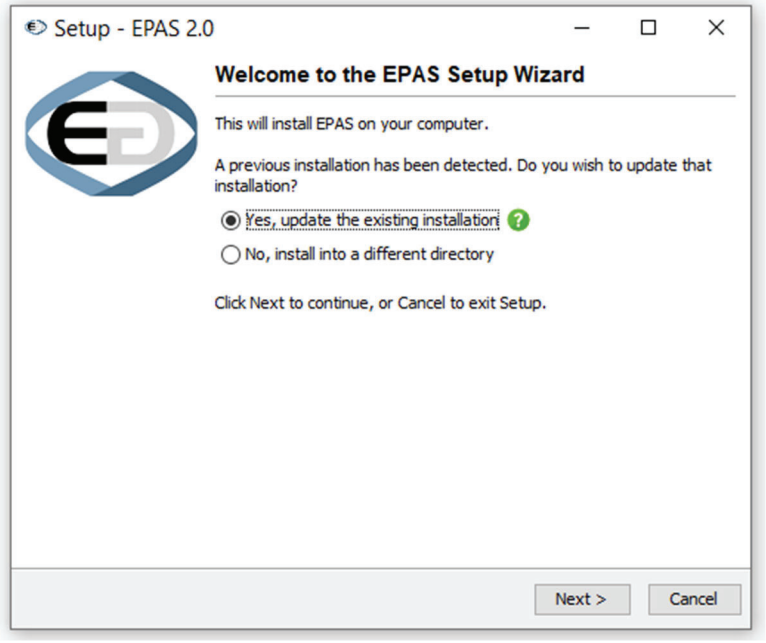
7. Wait for the Setup Wizard to launch.
8. If Windows Defender is installed on the PC, the installer may ask to confirm installation of the application on the hard drive. Select Yes to continue.
9. Click Next in the EPAS 2.0 Setup Wizard.

Figure 5. EPAS 2.0 Setup Wizard



10. If another version of EPAS™ is already installed on the computer (i.e., EPAS™ is being re-installed or being updated to a newer version), EPAS™ will prompt whether to install EPAS™ in the same directory and update the existing files OR to select a different directory.
    a. Click Yes to re-install / update the existing EPAS™ application; or
    b. Click No to designate a new directory to install EPAS™.

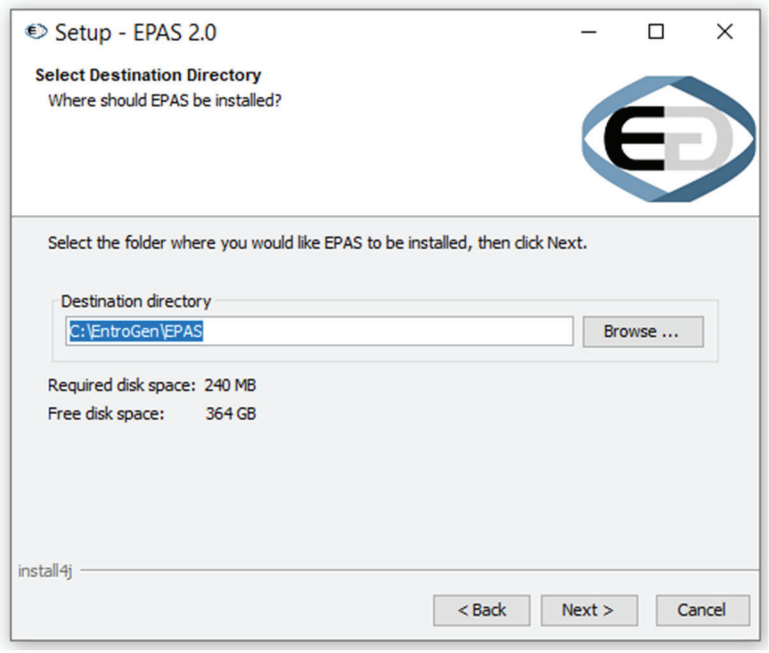
Figure 6. Re-installing EPAS™



Note: Updating EPAS™ will NOT erase any existing analysis files, user information, or traceability data stored in the existing directory. All existing data stored in previous versions of EPAS™ will be preserved.

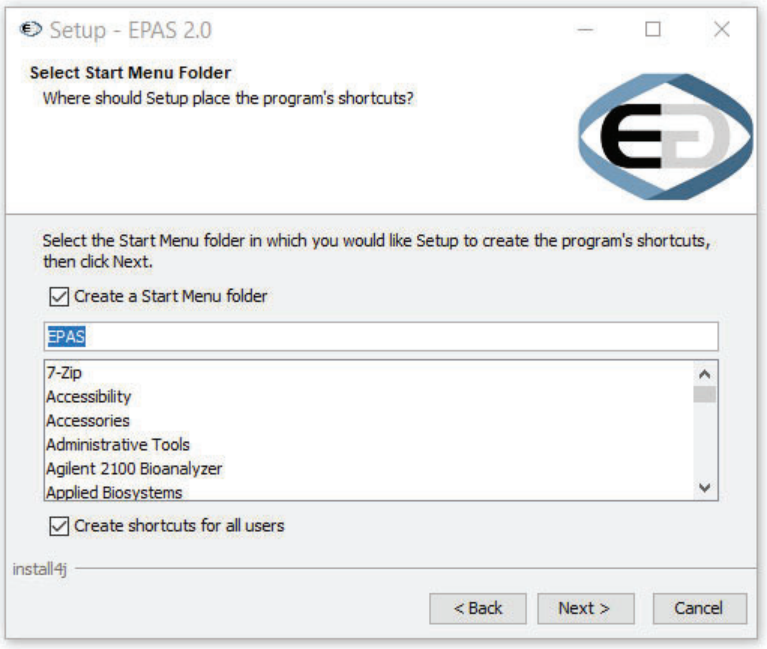
11. Select the Destination Directory where EPAS™ will be installed and click Next.

Figure 7. Select the EPAS™ Destination Directory



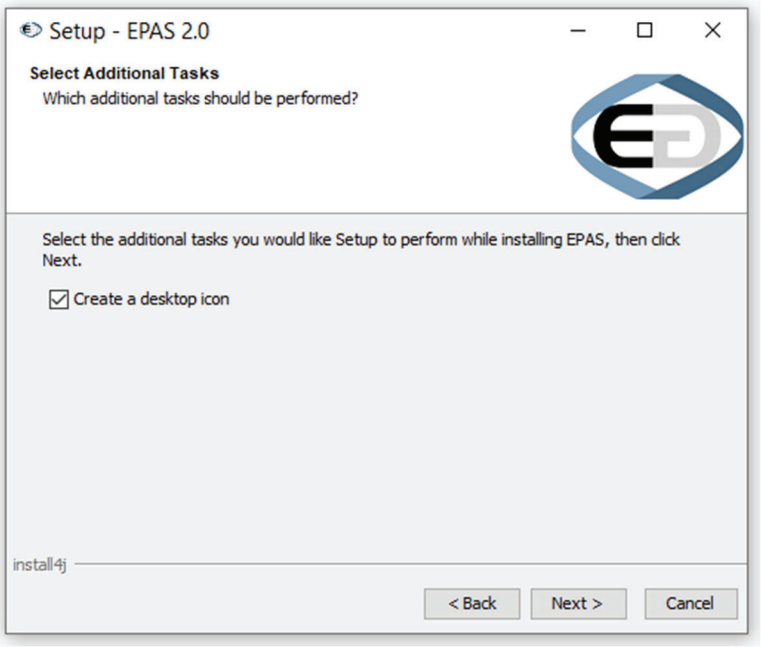
12. Check the boxes to create a Start Menu folder and / or shortcuts for all users. Uncheck the boxes if those features are not desired. Click Next to continue.

Figure 8. Creating Shortcuts



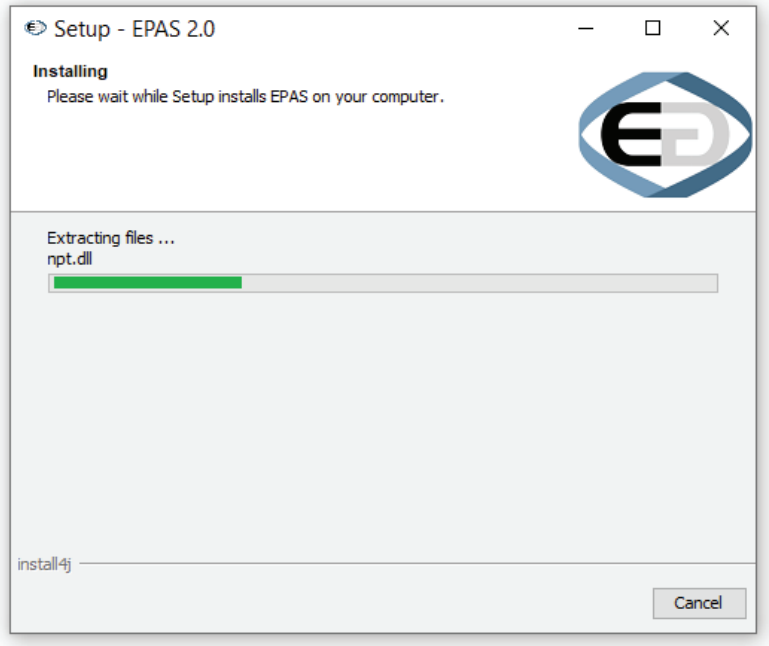
13. Check the box to create an EPAS™ desktop icon shortcut. Uncheck the box if this feature is not desired. Click Next to continue.

Figure 9. EPAS™ Desktop Icon Shortcut



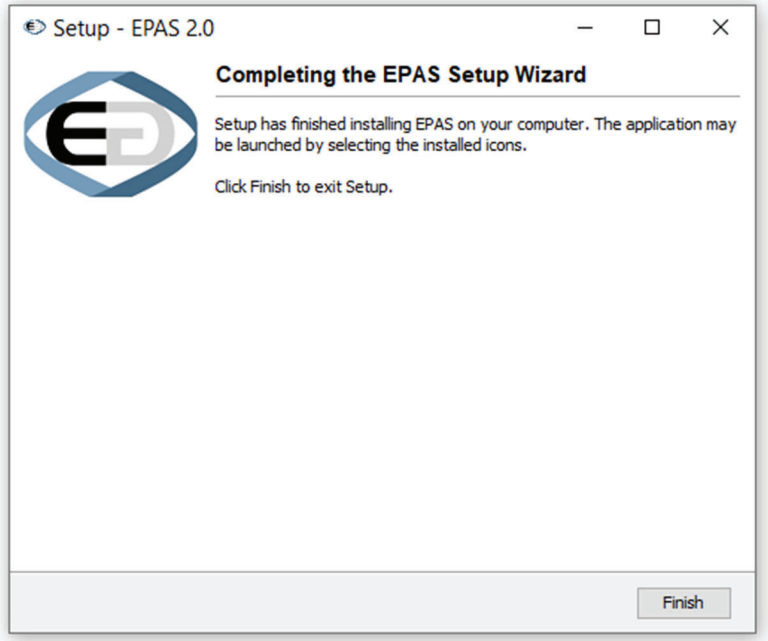
14. EPAS™ will begin to install.
15. Wait until the Setup Wizard has completed the installation process.

Figure 10. EPAS™ Installing on Computer



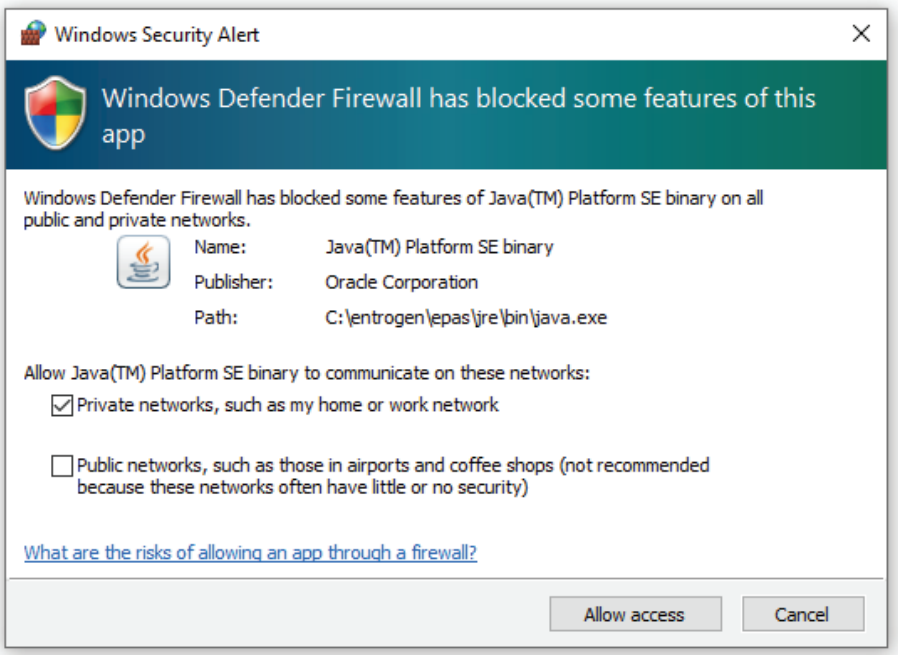
16. The EPAS Installation Wizard will prompt when installation is complete. Click Finish to exit the EPAS Installer.

Figure 11. EPAS™ Installation Complete



17. Once installed, launch EPAS™ from the desktop icon or from the destination folder by double-clicking on the EPAS.exe icon.
18. If a firewall notification is presented, select **Allow access**.

Figure 12. Firewall Access Notification



Notice to user: EntroGen requires its users receiving the encrypted USB drive to certify (by signing a document provided with the USB drive) that the older USB drive (with the outdated version of EPAS)

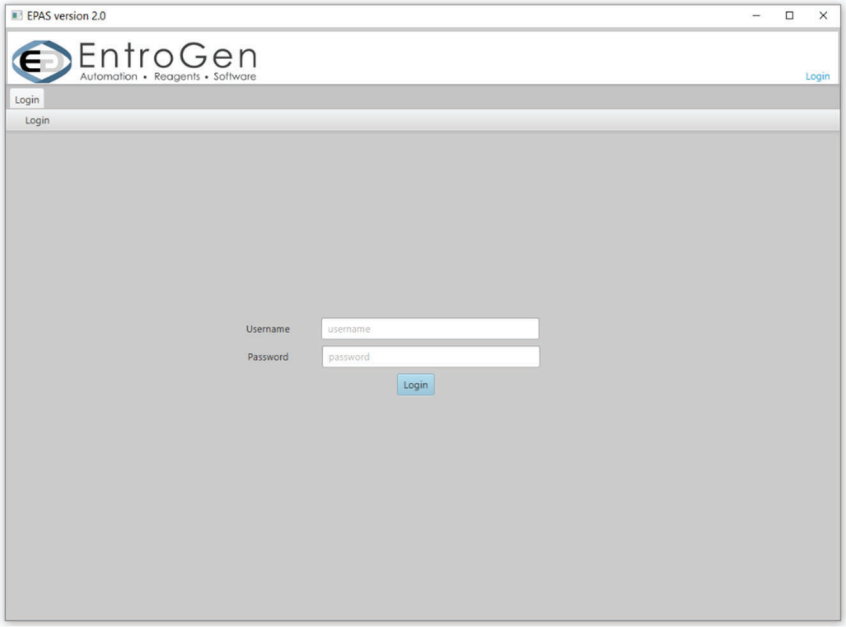
was either destroyed or returned to EntroGen. Users should store the USB drive according to their document and software retention policy, store them in a secure location. Users should not use the USB drives for other purposes.

10. Setting Up EPAS™

10.1. Initial EPAS™ Login

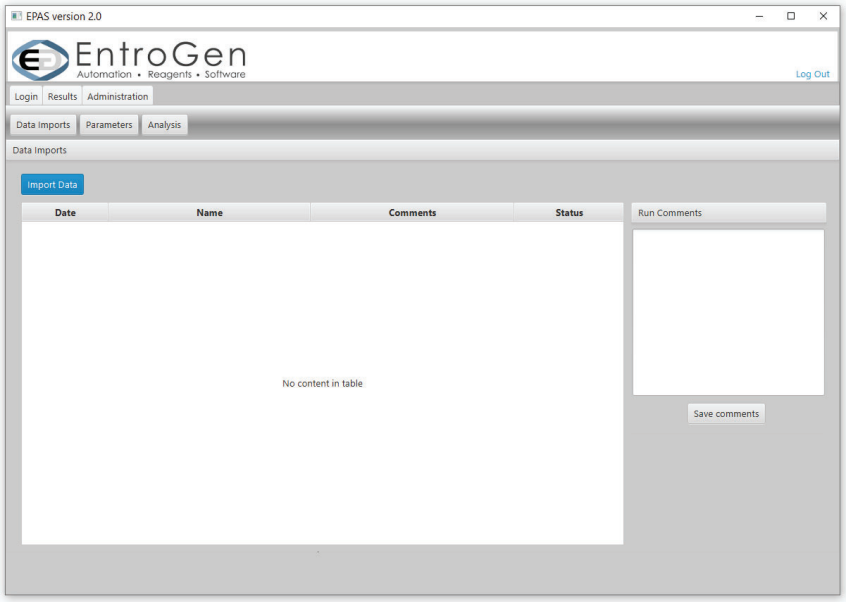
1. Launch the EPAS™ application.
2. Use the default login credentials supplied EntroGen for the initial login to EPAS™.
3. Enter the Username and Password and click Login.

Figure 13. EPAS™ Login Page



4. A successful login will direct the user to the Data Imports Page.

Figure 14. User Directed to Data Imports Page after Successful Login



5. Failed login attempts due to an incorrectly entered username or password will cause EPAS™ to display an error message.
    a. All users are limited to 4 total login attempts before EPAS™ temporarily inactivates the user account for 60 minutes (1 hour).
    b. After the temporary inactivation, the user will have 1 more attempt to login.
    c. If the user fails the final login attempt, EPAS™ will indefinitely inactivate the user account.
    d. A user with administrative privileges is required to reactivate an inactive user account.
6. It is recommended to create additional administrative, supervisory, and standard user accounts through the Administration – Users Management Page (see Section 10.2 for details).

10.2. Accessing the Users Management Tab

Only users with administrative level access privileges can add new user accounts and edit or remove existing user accounts.

1. Launch and log-into EPAS™.
2. Click on the **Administration** tab button located in the Navigation Menu.

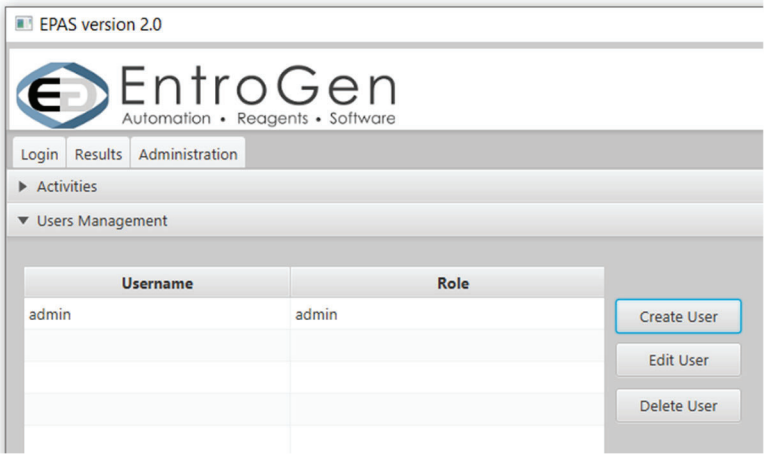
Figure 15. Administration Page Button in Navigation Menu

3. Click on the Users Management Tab from the list of Tabs displayed on the Administration Page.

10.2.1. Creating New User Accounts

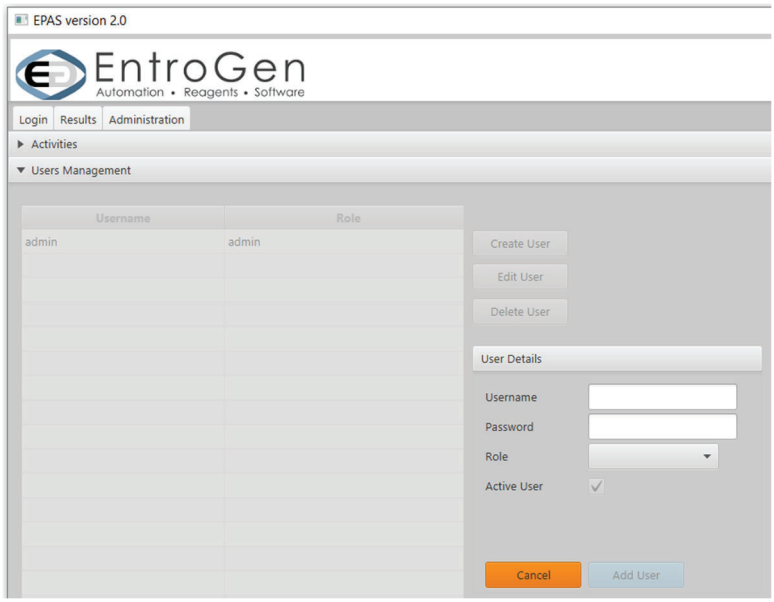
1. Click on the **Create User** button.

Figure 16. Administration Page - Users Management Tab



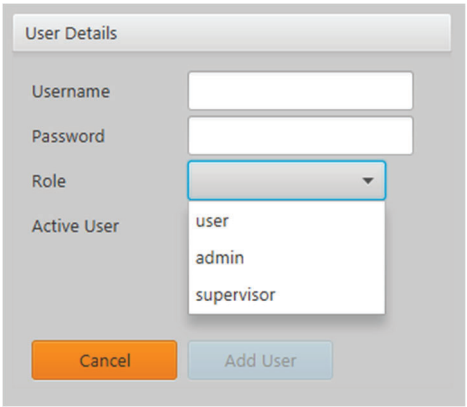
2. A User Details section will appear.
    a. When the User Details section is open, the Users Table and functional buttons become inactive. This is to ensure only one user account is created / edited at a time.
    b. The Add New user button will also be inactive until a password, username, and role are entered for the new user account.

Figure 17. Creating a New User - User Details



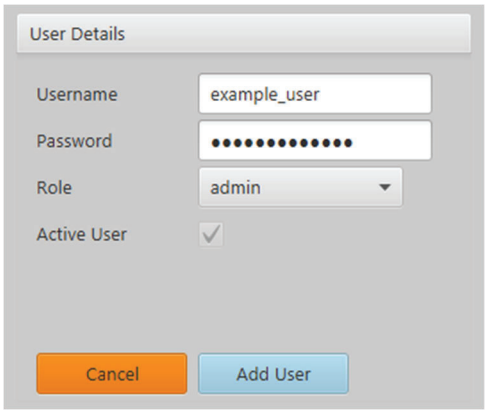
3. Enter a Username that is between 5-16 alphanumeric characters long (underscores and dashes are allowed, but other punctuation marks are not).
4. Enter a Password that is between 8-32 alphanumeric characters long and meets the following criteria:
    a. At least one uppercase letter.
    b. At least one lowercase letter.
    c. At least one number.
5. Select a user role from the drop-down menu: admin, supervisor, user (see Section 8.9.2).

Figure 18. Selecting User Role Designations for New Users



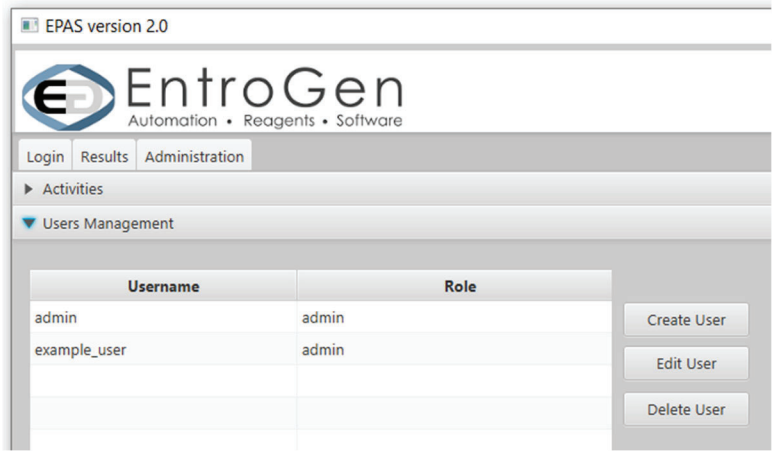
6. Check the Active User checkbox to activate the account or uncheck the checkbox to inactivate the account. Inactive accounts will not be able to login to EPAS™.
7. Once a valid username / password has been entered and a role designation has been selected, the Add User button will become active.
8. Click the **Add User** button to create the new user account or click the **Cancel** button to discard the new user details.

Figure 19. Creating a New User



9. When a new user is successfully created, the Username and Role will appear in the Users Table under the Users Management Tab.

Figure 20. Successful Creation of a New User Account

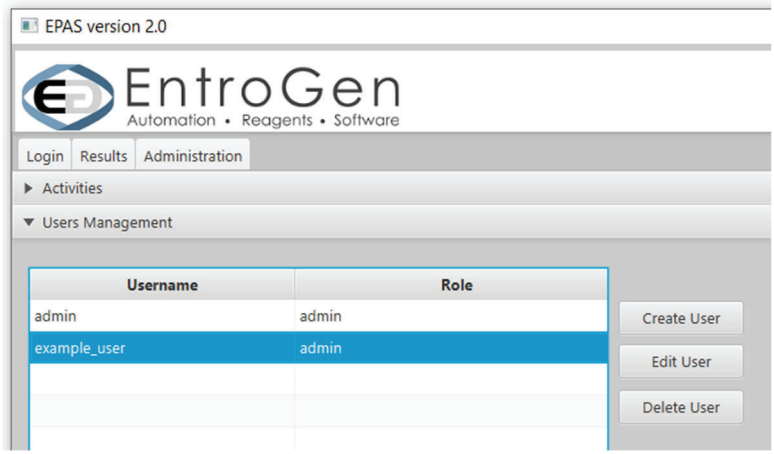


10.2.2. Editing Existing User Accounts

Only users with administrative level access privileges can add new user accounts and edit or remove existing user accounts.

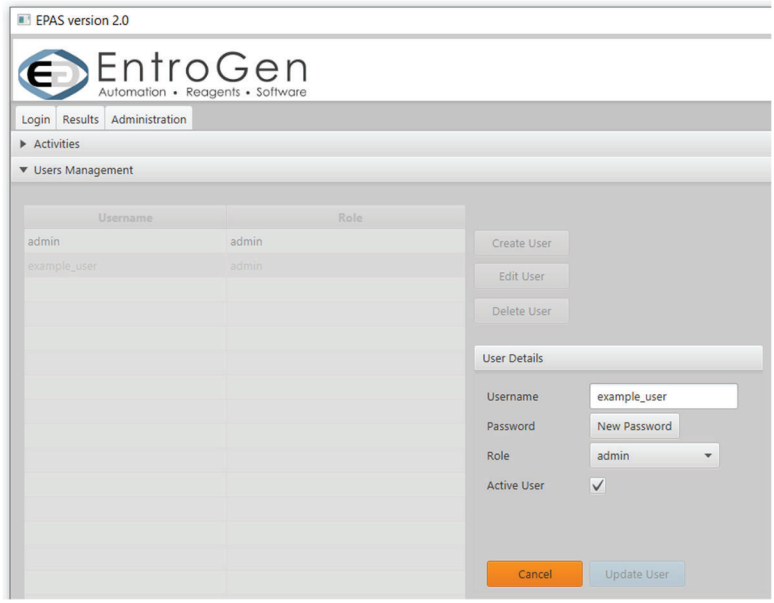
1. Select a user account by clicking on the user entry in the Users Table.
2. Click the **Edit User** button.

Figure 21. Selecting a User to Edit



3. An Edit User Details section will appear.
    a. When the User Details section is open, the Users Table and functional buttons become inactive. This is to ensure only one account is edited at a time.
    b. The Update User button will also be inactive until changes are made.
    c. The User Details section will be populated with the selected user's account details.

Figure 22. Edit User Details

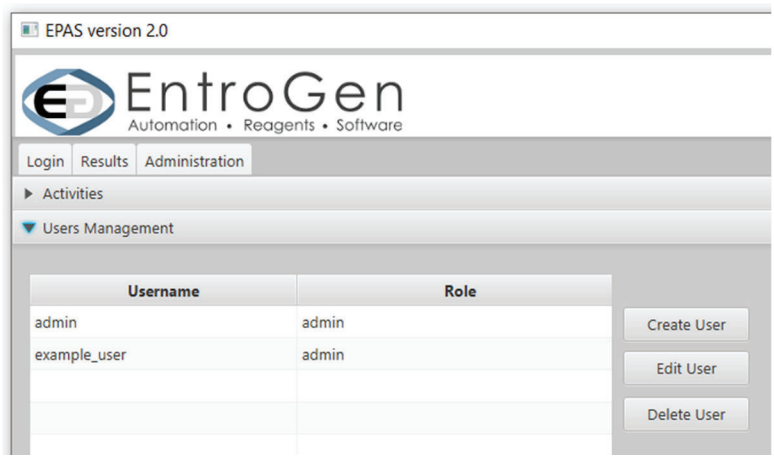


4. Edit the user details.
5. A new username must be between 5-16 alphanumeric characters long (underscores and dashes are allowed, but other punctuation marks are not).
6. To reset the password, click the **New Password** button.
7. Enter a Password that is between 8-32 alphanumeric characters long and meets the following criteria:

a. At least one uppercase letter.
b. At least one lowercase letter.
c. At least one number.

8. Select a user role from the drop-down menu: admin, supervisor, user (see Section 8.9.2).
9. Check the Active User checkbox to activate the account or uncheck the checkbox to inactivate the account. Inactive accounts will not be able to login to EPAS™.
10. When any change is made to the existing user account, the Update User button will become active.
11. Once all desired changes are made, click the **Update User** button to save the changes to the EPAS™ database.

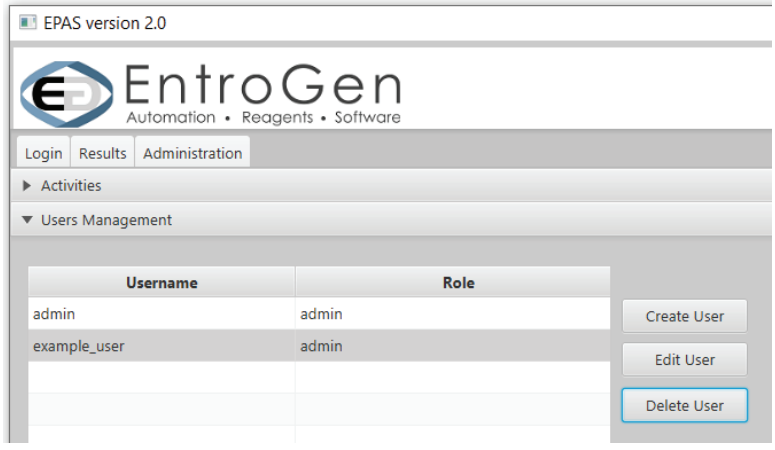
Figure 23. Successful Modification to an Existing User Account



10.2.3. Deleting an Existing User Account

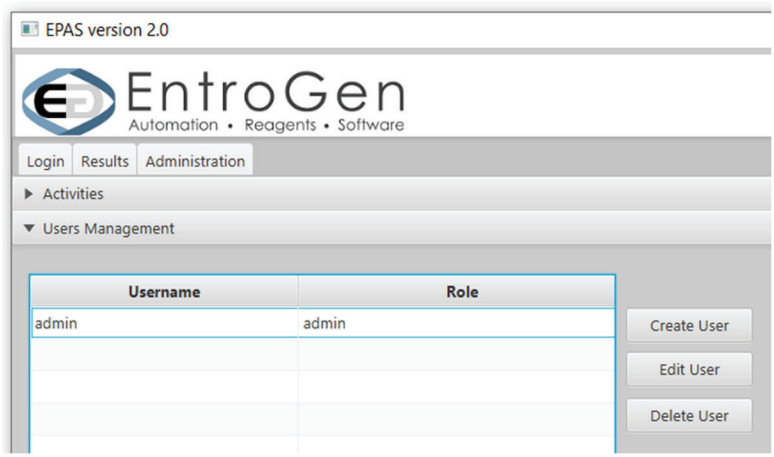
1. In the Users Management Tab on the Administration Page, select a user account.
2. Click the **Delete User** button.

Figure 24. Deleting an Existing User Account



3. EPAS™ will display confirmation message prior to deleting the selected user account because a deletion is irreversible.
4. Clicking Yes will cause EPAS™ to delete the user account, whereas clicking No will retain the user account and return to the User Management Tab.
5. A successful deletion will remove the selected user account from the Users Table.

Figure 25. Successful User Deletion



Note: A user that is currently logged into an active session of EPAS™ cannot delete themselves. EPAS™ will display an error message and prevent the deletion from occurring.

11. Viewing and Exporting User Activities

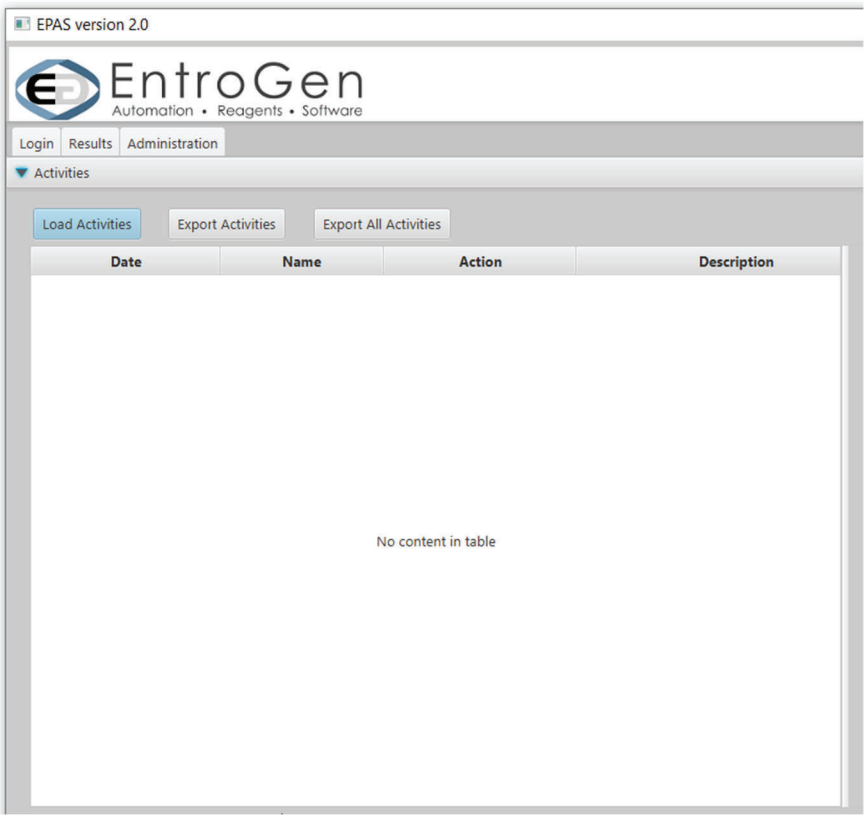
Only users with administrative or supervisory level access can view and export user activities in EPAS™.

1. Launch and log into EPAS™.
2. Click on the **Administration** tab button located in the Navigation Menu.

Figure 26. Administration Page Button in Navigation Menu

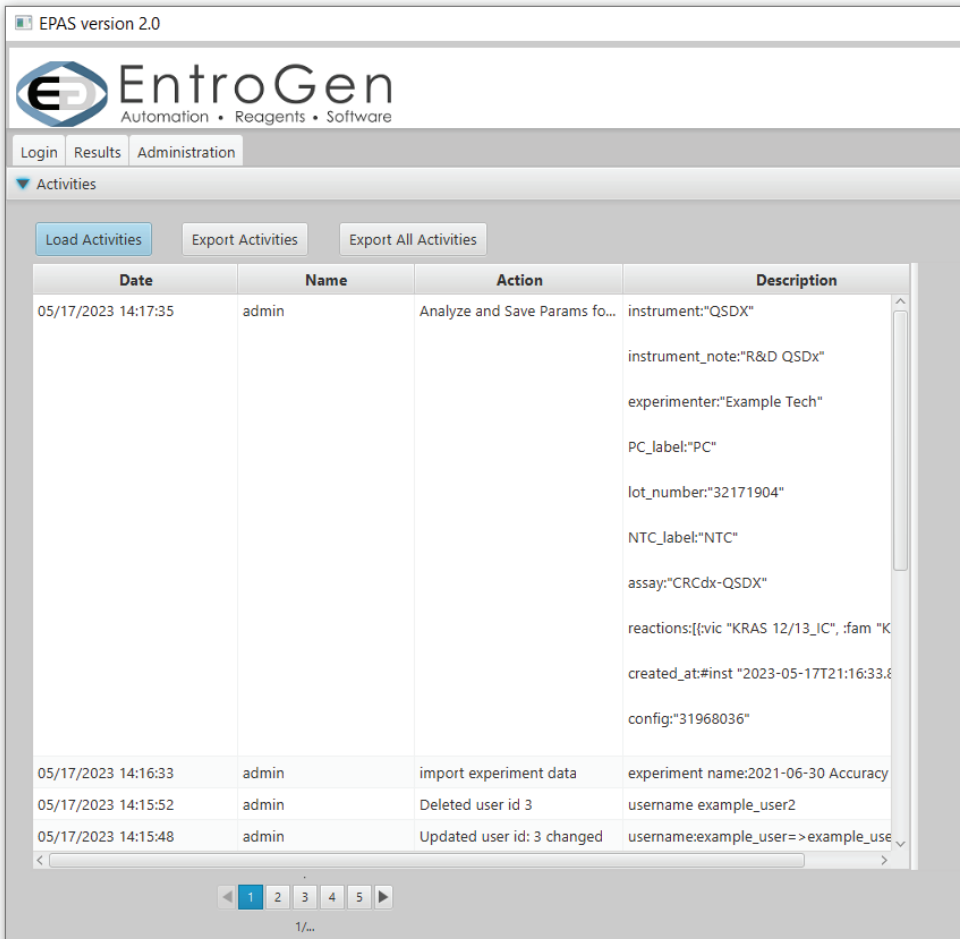
3. Click on the Activities Tab from the list of Tabs displayed on the Administration Page.

Figure 27. Administration Page - Activities Tab



4. Click on the **Load Activities** button to view all recorded user activities.

Figure 28. Load Activities



Note: EPAS™ is programmed to keep track of all user activities (such as creation / deletion / activation / inactivation / editing of users, data file imports / deletions, analysis parameters, data analysis, report generation, data exports, and user login / log outs).

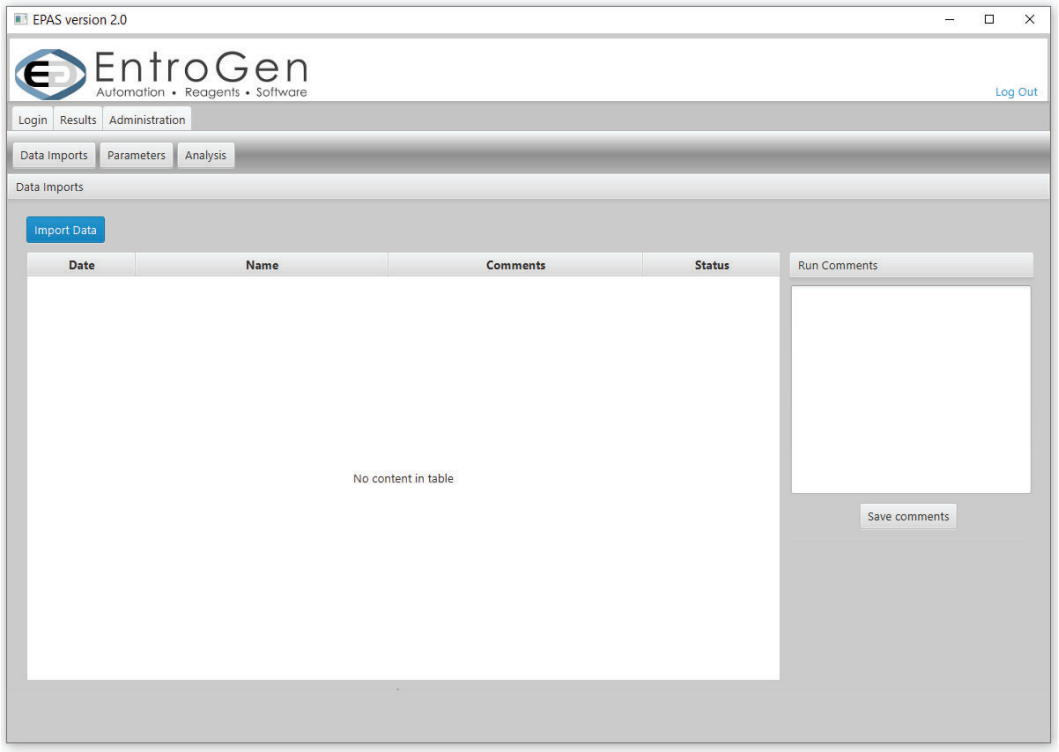
5. Use the vertical and horizontal scroll bars to view the data shown in the Activities Table.
6. Use the Page Scroll buttons underneath the Activities to scroll between table pages. The blue highlighted Page Scroll button represents the currently displayed page.
7. The **Export Activities** button will export the currently displayed page as a PDF.
8. The **Export All Activities** button will export all the recorded activities as a PDF.

12. Importing and Analyzing CRCdx® Data Files with EPAS™

12.1. Importing CRCdx® Data Files into EPAS™

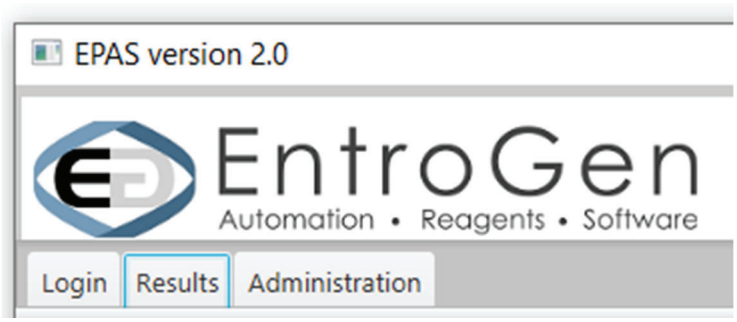
1. Launch and log into EPAS™.

Figure 29. User Directed to Data Imports Page after Successful Login



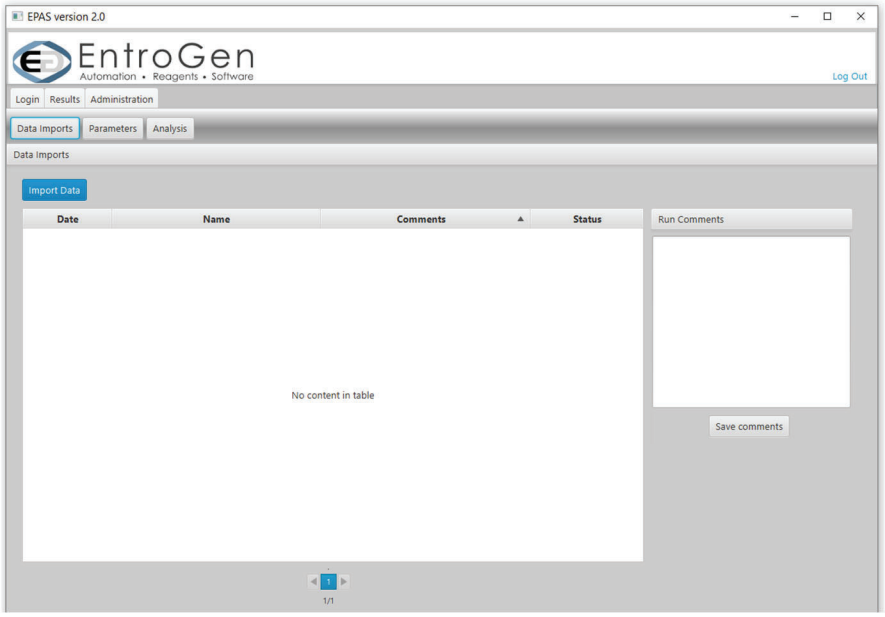
2. To navigate to the Data Imports Page from any other EPAS™ page, click the **Results** button in the Navigation Menu (located underneath the EntroGen logo).

Figure 30. Results Page Button in Navigation Menu



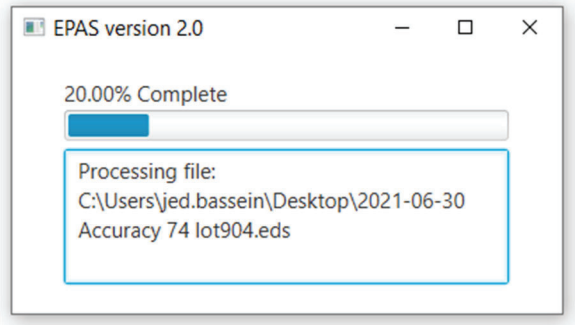
3. Then click on the **Data Imports** button in the Results Navigation Menu (located underneath the Navigation Menu).

Figure 31. Data Imports Button in Results Navigation Menu



4. Click the **Import Data** button.
5. Navigate to the location where the CRCdx® data file is stored.
6. Select the data file and click **Open**.
7. EPAS™ will display a Data Import Progress Window that shows the status of the import process.

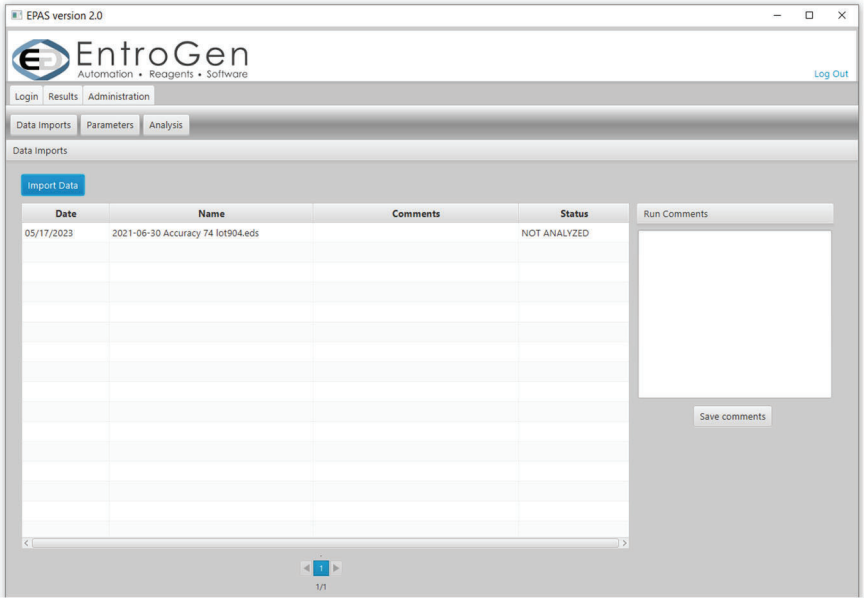
Figure 32. Data Import Progress Window



Note: EPAS™ will only accept .eds data files that were produced in the IVD mode of the QSDx instrument using the CRCdx® Test Definition Document (TDD) file. If the selected data file does not meet these criteria, then EPAS™ will produce an error message and prevent the data import process from completing.

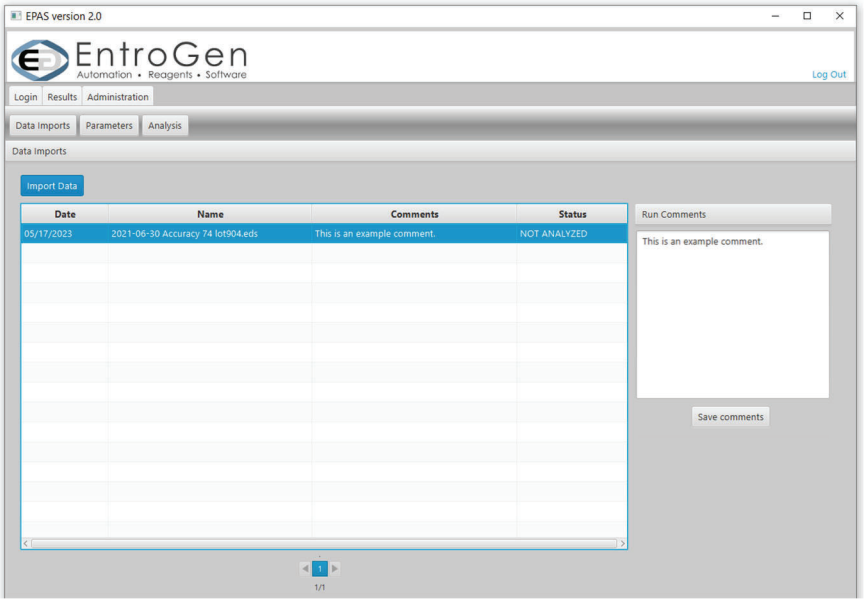
8. Once complete, the Data Import Progress Window will close and the data file will appear in the Imports Table.

Figure 33. Imported CRCdx® Data File



9. Page Scroll buttons are displayed below the Imports Table and can be used to navigate between pages in the table.
10. EPAS™ will display the date the data file was imported, the file name, any comments associated with the file, and the status.
    a. Not Analyzed means the file has not been analyzed by EPAS™.
    b. Analyzed the file has been analyzed by EPAS™.
11. To annotate a data file, select the data file, type the comments into the Run Comments Text Box, and click the **Save Comments** button.
    a. Run comments will be displayed in the Comments Column after being saved.

Figure 34. Run Comments Saved to Imported Data File

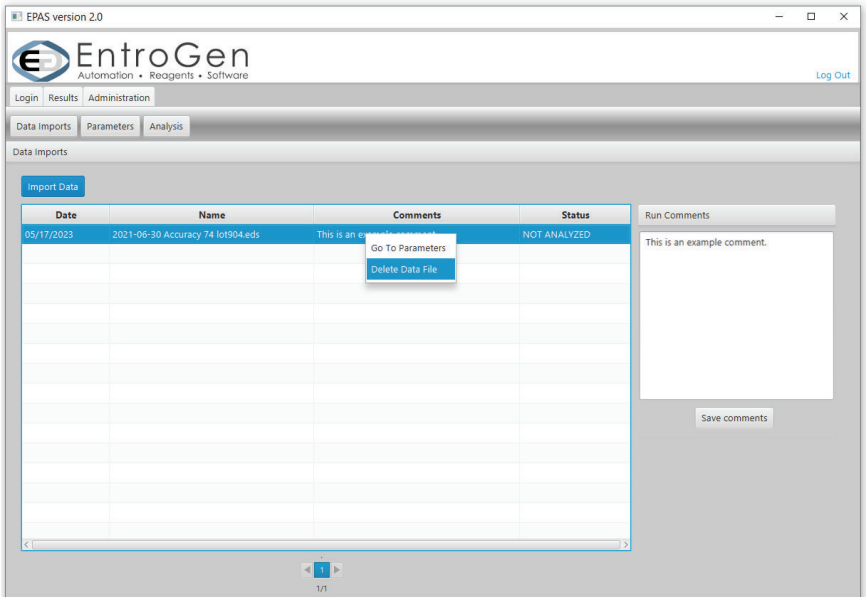


12.2. Deleting Data Files from EPAS™

Only Admin level users have the ability to delete data files from EPAS™.

1. To delete a data file, right-click on the data file.
2. Select the **Delete Data File** option.

Figure 35. Selecting a Data File for Deletion

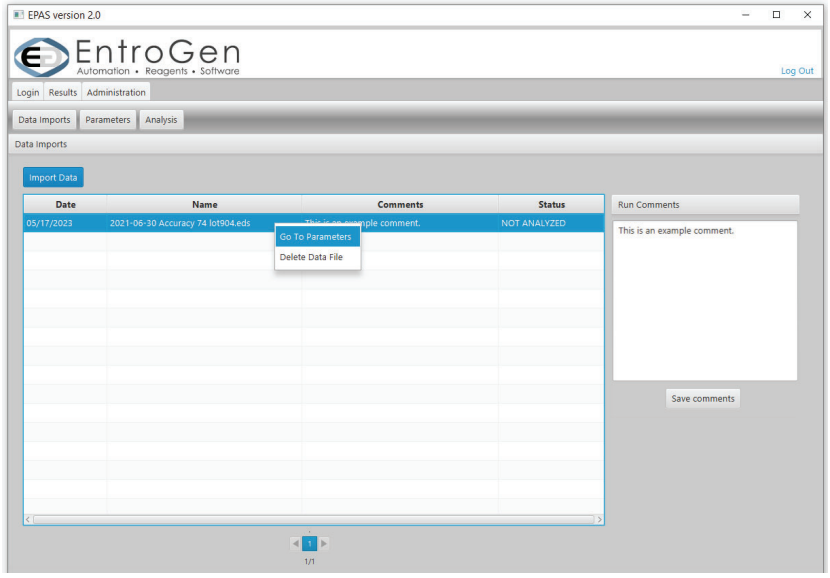


3. EPAS™ will display a confirmation message prior to deleting the selected data file because a deletion is irreversible.
4. Clicking Yes will cause EPAS™ to delete the data file, whereas clicking No will retain the data file and return to the Data Imports page.

13. Inputting Traceability Parameters and Assigning Control Samples

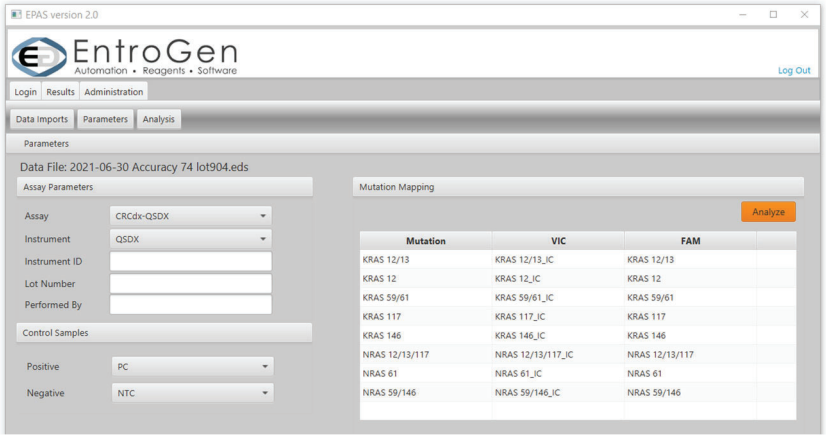
1. Double-click on the file or right-click on the file and select **Go To Parameters** to load a data file for analysis.
    a. *Note: If the data file is not properly loaded, EPAS™ will not analyze the results.*

Figure 36. Loading a Data File into the Parameters Page.



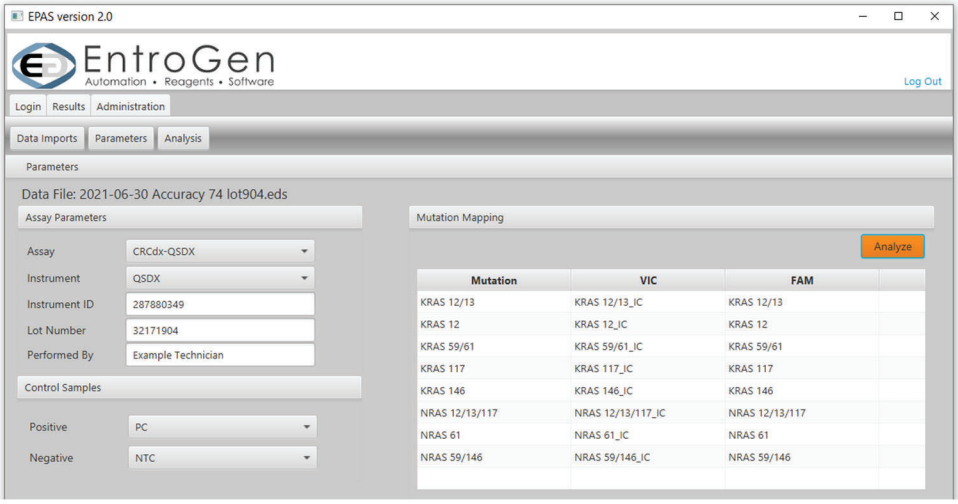
2. When a data file is loaded into the Parameters page, EPAS™ will display the file name at the top of the Parameters page.
3. EPAS™ will automatically populate the Assay and Instrument drop-down menu selections.
4. EPAS™ will also automatically select the Positive (PC) and Negative (NTC) control samples.
    a. Manually select the Positive and /or Negative Controls if they are not properly named.
    b. A Positive and Negative Control must be included on every CRCdx® plate. If a PC and NTC are not available or if the wrong samples are chosen for the PC / NTC, then EPAS™ will fail the plate and the results will be invalid.
    c. The same sample cannot be used for the PC & NTC selection. Unique samples must be chosen for each control.

Figure 37. Results - Parameters Page with a Properly Loaded Data File



5. Enter the Instrument ID for the QSDx.
6. Enter the CRCdx® assay lot number.
7. Enter who is performing the analysis.

Figure 38. Data File with Entered Traceability Parameters and Assigned Control Samples Ready for Analysis



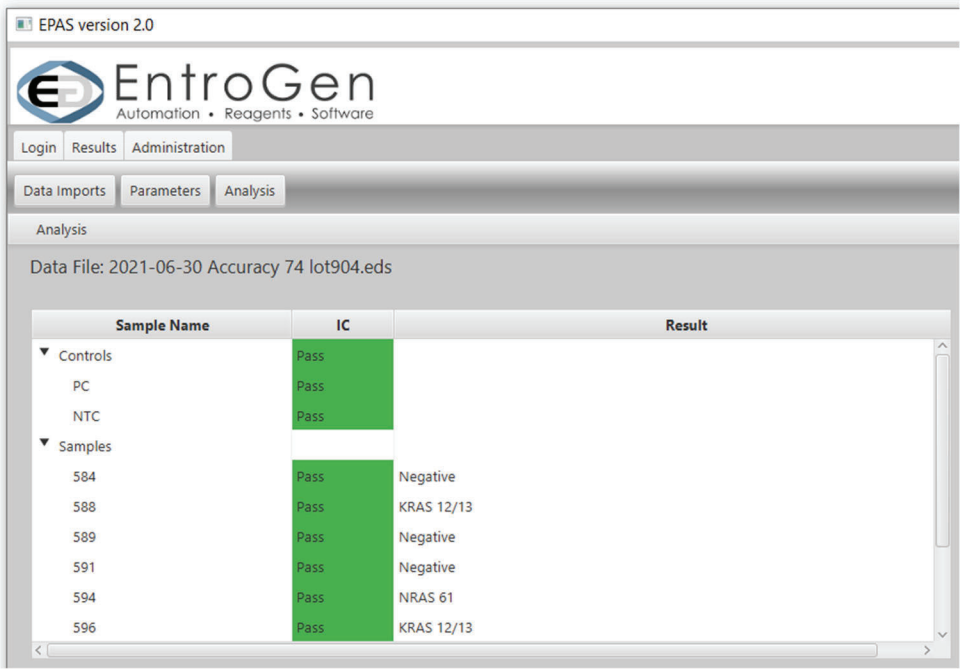
8. Click the **Analyze** button.
9. EPAS™ will display a confirmation message
10. Confirm if the entered data is correct.
    a. *Note: After clicking Yes, EPAS™ will undergo a series of internal data verification steps to ensure the loaded data file conforms to CRCdx® data requirements and all required configuration files are installed and valid. If EPAS™ encounters and error, a message will display and data analysis will not proceed.*
11. If all data validation and verification steps pass, EPAS™ will direct the display to the Analysis Page to view the results.

14. Viewing EPAS™ Analysis Results and Report Generation

14.1. Viewing Results

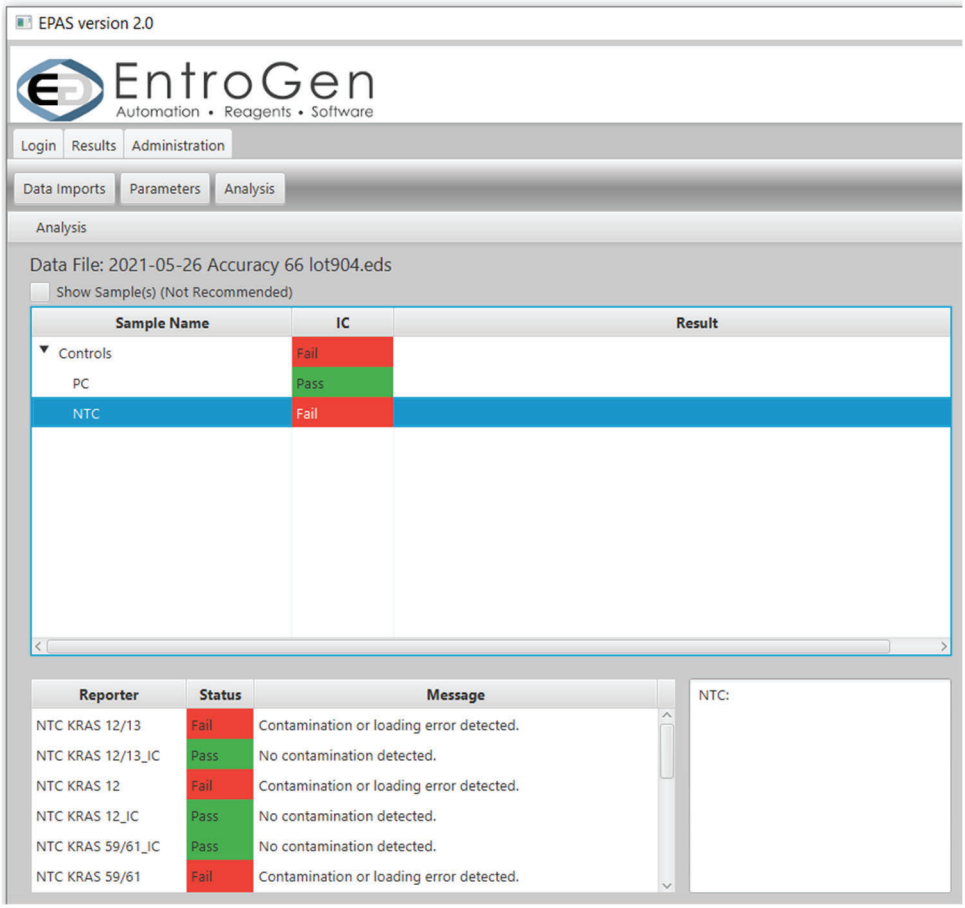
1. The **Analysis** tab will display the analytical results for the controls and each sample tested.

Figure 39. EPAS™ Report Table Displaying Results Analysis



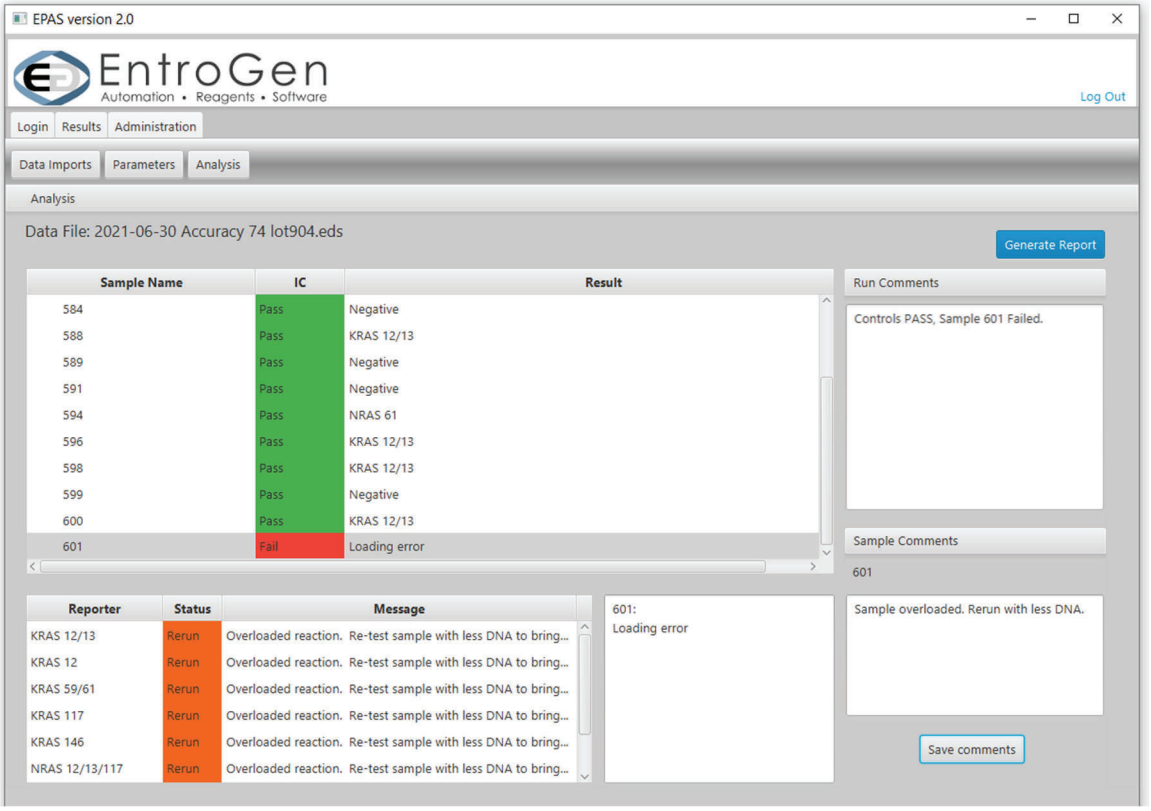
2. Sample Names are displayed in two (2) groups:
    a. Controls: Positive (PC) and Negative (NTC) must be defined in the “Parameters” Page.
    b. Samples: Unknown CRC FFPE Samples tested by CRCdx® RAS assay.
3. The IC column indicates the PASS / FAIL status of the internal control values for the tested “Controls” and “Samples”.
4. If a Control’s IC status Fails, then sample results will not be visible. To over-ride and view the sample details check the box next to “Show sample? (Not Recommended)” at the top of the window.
    a. Failed controls will invalidate all run results and require all samples to be re-run. Results from plates with failed controls must not be reported. See Figure 40 and Section 14.2 below for details.
    b. Failed samples (with passing controls) require will require a rerun of the sample and must not be reported as positive or negative. However, other samples on the plate with valid IC status can still be reported. See Figure 41 and Section 14.2 below for details.
5. If the PC and NTC PASS, continue with analysis, interpretation, and test report generation.

Figure 40.Example of a CRCdx® Run with Failed Controls



6. To view more details about a Control or Sample (not shown above), select the Control or Sample of interest under the **Sample Name** Column by clicking on it.
    a. Information about the **Reporter**, **Status**, and **Message** results will appear in the Status Table located below the Reporter Table.
    b. Clicking on or selecting a reporter will cause more information, such as the **Ct values** to display in the Message Display Box which is located below the Report Table and to the right of the Status Table (see Figure 41 above).
7. Each sample is evaluated based on raw fluorescence to ensure liquid is present in the well and based on the Ct values reported for VIC and FAM. The samples results are reported according to the acceptance criteria and analytical cut-off values described in Table 9 below.
8. Run and Sample Comments can be entered in their respective text boxes that appear to the right of the Report and Status Tables.
9. Clicking the **Save Comments** button will save the entered text to the EPAS database.
    a. *Note: These comments will be produced on the final report generated for the results (See Section 14.3 below for details).*

Figure 41. Specimen Status and Run / Sample Comments



10. To create / generate a Test Report that contains the Analysis Results:
    a. Click the **Generate Report** button in the Analysis window.
    b. Name and select a known file location where to save the report.
    c. Reports can be saved either as an .xlsx (default option) or .csv.

14.2. Retesting Samples and Plates with Invalid Results

1. Samples on plates / runs that fail PC or NTC requirements must be retested.
    a. A control (PC / NTC) failure can indicate an error in reagent handling or contamination of reagents.
    b. ALL data from a plate / run with a failed control is INVALID. Reagent master mixes must be remade and all samples must be rerun.
2. Samples that fail due to loading errors must be retested.
    c. Assess the VIC values for each reaction to determine if the sample is over- (Ct < 24.0) or under-loaded (Ct > 26.5).
    d. EPAS™ will produce a message to assist in determining whether more or less DNA should be loaded to bring the sample within acceptable range.
    e. Adjust the concentration as necessary and proceed with retesting.
    f. If a VIC target cannot be reached for all reactions, the sample cannot be evaluated.

14.3. Interpreting the Test Report

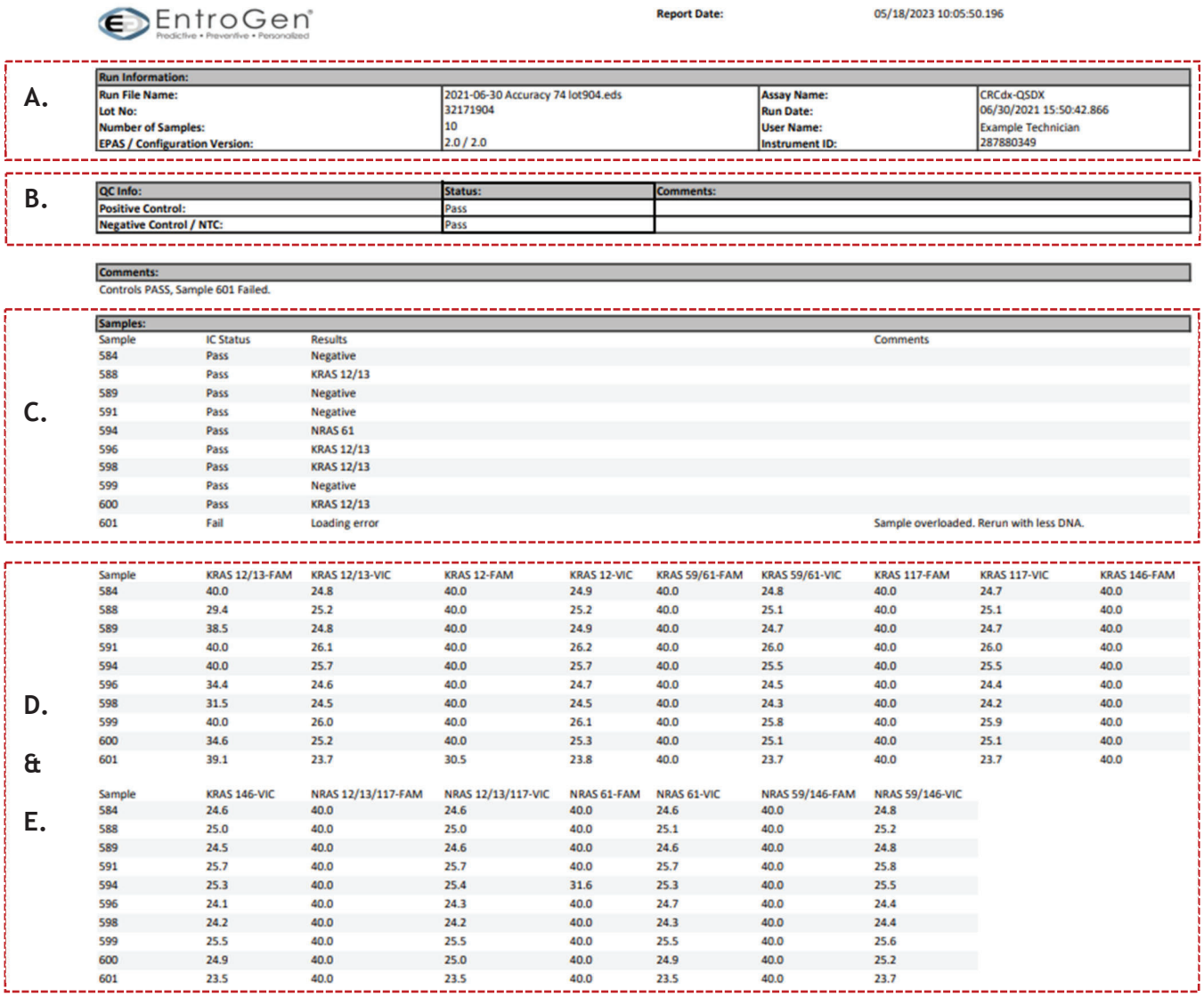
1. The Generate Results button produces an .xlsx (default) or .csv document that contains the results of the EPAS™ analysis.
2. If the VIC Ct value for the sample is between 24.0 – 26.5, the IC status of the sample is PASS.
    a. Mutations are reported as Negative (no mutation detected) or grouped into one (1) of eight (8) total reactions groups (see Table 6 below for details).

Table 6. EPAS™ Mutation Reporting Schematic

IC Status	EPAS™ Result	Interpretation
Pass	Negative	No Mutation Detected
Pass	KRAS 12/13	KRAS G12A, G12D, G12R, G12V, G13D
Pass	KRAS 12	KRAS G12C, G12S
Pass	KRAS 59/61	KRAS A59E, A59G, A59T, Q61H, Q61L, Q61R
Pass	KRAS 117	KRAS K117N, K117R, K117E
Pass	KRAS 146	KRAS A146T, A146P, A146V
Pass	NRAS 12/13/117	NRAS G12D, G12C, G12S, G13R, G13V, K117R
Pass	NRAS 61	NRAS Q61H, Q61L, Q61K, Q61R
Pass	NRAS 59/146	NRAS A59D, A59T, A146T
Fail	Loading error	Samples results are invalid due to one or more reactions being over/under-loaded or omitted. Retest the specimen sample following instructions in the Package Insert.
Fail	Empty lane	Results are invalid due to no reagent present in all wells. Occurs when a blank lane was not omitted from analysis in the QSDX software at run initiation. Abnormal Ct values may be displayed.
Fail	Empty control lane or well	Results are invalid due to no liquid being present in one or all wells associated with specified control. Occurs when an omitted / empty well is detected in a control lane.
Fail	--	Results are invalid due to failed control. Ct values outside of acceptance criteria range.

3. If the VIC Ct value for the sample is not between 24.0 – 26.5, the IC status of the sample is FAIL.
    a. Samples can FAIL if a well was omitted / missed and was not loaded with reagent.
    b. If the sample IC status is FAIL, the RAS mutation status cannot be determined.
    c. The sample must be re-run with more or less DNA (depending on the VIC Ct value)
        i. VIC Ct < 24.0 – Overloaded reaction. Re-test with less DNA to bring sample within acceptable range.
        ii. VIC Ct > 26.5 – Underloaded sample. Insufficient amount of DNA. Re-test sample with more DNA to bring VIC within acceptable range.
4. If either the Positive (PC) and / or Negative (NTC) control FAIL to meet acceptance criteria, all data produce on the plate is invalid.

Figure 42. Example of EPAS™ Generated Test Report (.xlsx)



EntroGen
Predictive • Preventive • Personalized

Report Date: 05/18/2023 10:05:50.196

A.

Run Information:			
Run File Name:	2021-06-30 Accuracy 74 lot904.eds	Assay Name:	CRCdx-QSDX
Lot No:	32171904	Run Date:	06/30/2021 15:50:42.866
Number of Samples:	10	User Name:	Example Technician
EPAS / Configuration Version:	2.0 / 2.0	Instrument ID:	287880349

B.

QC Info:	Status:	Comments:
Positive Control:	Pass	
Negative Control / NTC:	Pass	

Comments:
Controls PASS, Sample 601 Failed.

C.

Samples:

Sample	IC Status	Results	Comments
584	Pass	Negative	
588	Pass	KRAS 12/13	
589	Pass	Negative	
591	Pass	Negative	
594	Pass	NRAS 61	
596	Pass	KRAS 12/13	
598	Pass	KRAS 12/13	
599	Pass	Negative	
600	Pass	KRAS 12/13	
601	Fail	Loading error	Sample overloaded. Rerun with less DNA.

D.

Sample	KRAS 12/13-FAM	KRAS 12/13-VIC	KRAS 12-FAM	KRAS 12-VIC	KRAS 59/61-FAM	KRAS 59/61-VIC	KRAS 117-FAM	KRAS 117-VIC	KRAS 146-FAM
584	40.0	24.8	40.0	24.9	40.0	24.8	40.0	24.7	40.0
588	29.4	25.2	40.0	25.2	40.0	25.1	40.0	25.1	40.0
589	38.5	24.8	40.0	24.9	40.0	24.7	40.0	24.7	40.0
591	40.0	26.1	40.0	26.2	40.0	26.0	40.0	26.0	40.0
594	40.0	25.7	40.0	25.7	40.0	25.5	40.0	25.5	40.0
596	34.4	24.6	40.0	24.7	40.0	24.5	40.0	24.4	40.0
598	31.5	24.5	40.0	24.5	40.0	24.3	40.0	24.2	40.0
599	40.0	26.0	40.0	26.1	40.0	25.8	40.0	25.9	40.0
600	34.6	25.2	40.0	25.3	40.0	25.1	40.0	25.1	40.0
601	39.1	23.7	30.5	23.8	40.0	23.7	40.0	23.7	40.0

&

E.

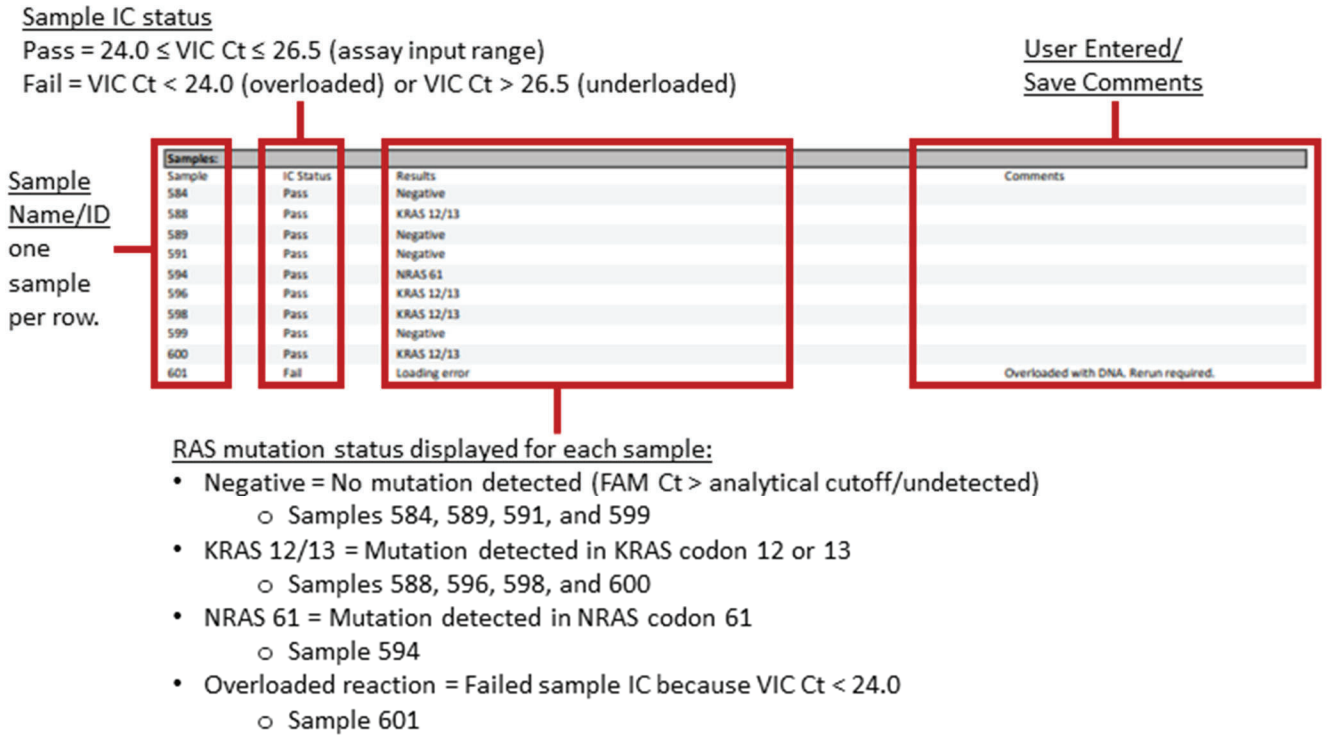
Sample	KRAS 146-VIC	NRAS 12/13/117-FAM	NRAS 12/13/117-VIC	NRAS 61-FAM	NRAS 61-VIC	NRAS 59/146-FAM	NRAS 59/146-VIC
584	24.6	40.0	24.6	40.0	24.6	40.0	24.8
588	25.0	40.0	25.0	40.0	25.1	40.0	25.2
589	24.5	40.0	24.6	40.0	24.6	40.0	24.8
591	25.7	40.0	25.7	40.0	25.7	40.0	25.8
594	25.3	40.0	25.4	31.6	25.3	40.0	25.5
596	24.1	40.0	24.3	40.0	24.7	40.0	24.4
598	24.2	40.0	24.2	40.0	24.3	40.0	24.4
599	25.5	40.0	25.5	40.0	25.5	40.0	25.6
600	24.9	40.0	25.0	40.0	24.9	40.0	25.2
601	23.5	40.0	23.5	40.0	23.5	40.0	23.7

A. Run Information: displays traceability parameters used to analyze the CRCdx® data file.
   i. Run File Name: displays the file name for the imported CRCdx® data file.
   ii. Lot No: displays the CRCdx® lot number entered in the Parameters page.
   iii. Number of Samples: displays the total number of samples analyzed on the CRCdx® plate.
   iv. EPAS / Configuration Version: displays the version of EPAS™ and the version of the configuration file used to analyze the CRCdx® file.
   v. Assay Name: displays the assay name for the assay configuration file used to analyze the CRCdx® plate.
   vi. Run Date: displays the date the CRCdx® data file was created.
   vii. User Name: displays the Performed By value entered in the Parameters page.
   viii. Instrument ID: displays the instrument ID value entered in the Parameters page.

B. QC Info: displays the quality control (QC) metrics determined for the Positive (PC) and Negative (NTC) control samples assigned in the Parameters page.
   i. If Status is PASS for both controls, the run is valid.
   ii. If Status is FAIL for either or both controls, the run is invalid.
   iii. Comments: any comments saved in the Sample comments text box in the Analysis page will appear here.

C. Sample Mutation Status Section: reports the PASS / FAIL result for the IC status and the RAS mutant results determined by EPAS™ (see Figure 43 below for more details).

Figure 43. C. Sample Mutation Status Section



D. Raw Ct Values Section: displays the FAM and VIC Ct values obtained for each CRCdx® reaction and sample tested in the analyzed CRCdx® data file (see Figure 44 below for more details).

Figure 44. D. Raw Ct Values Section

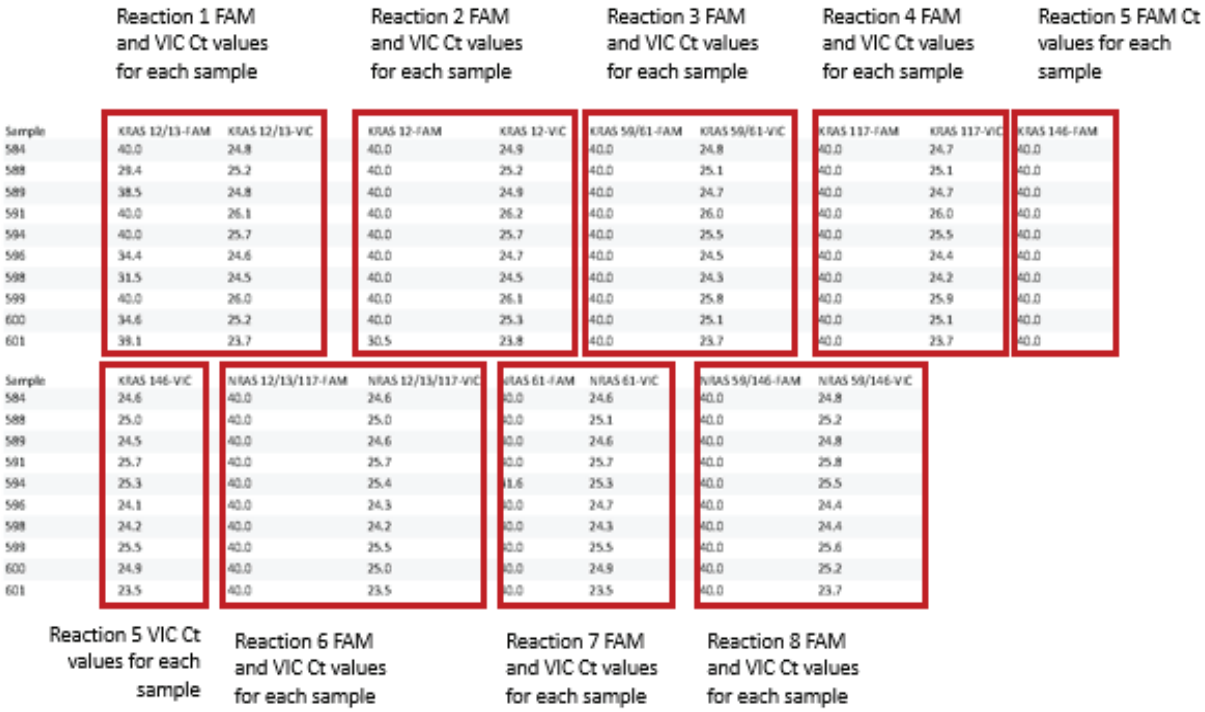
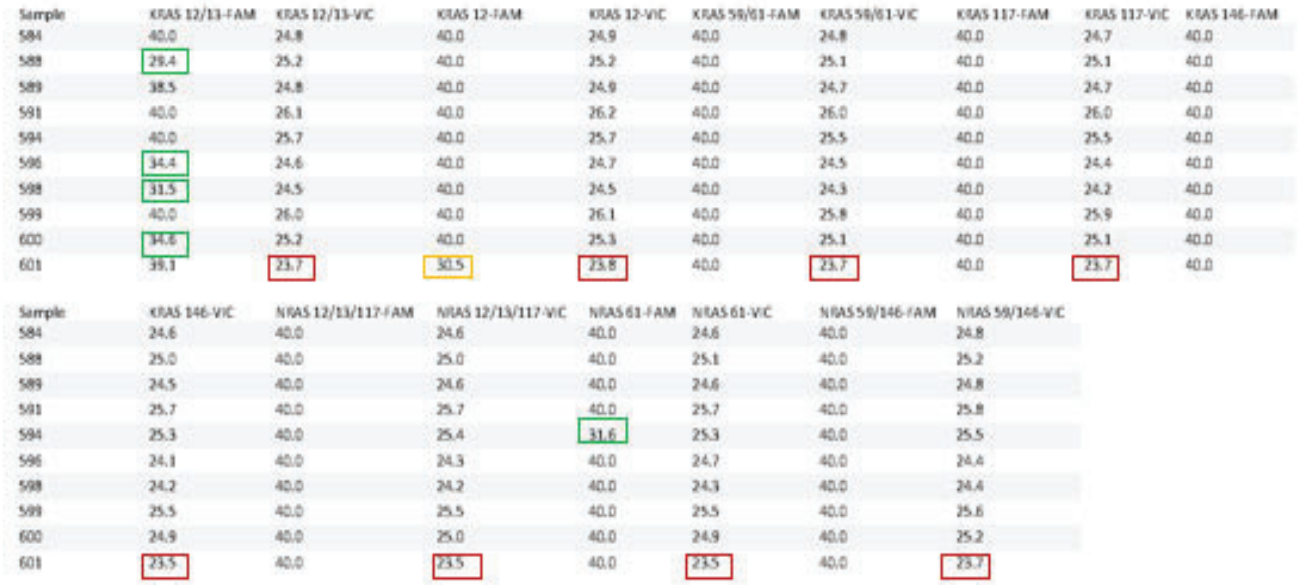


Figure 45. Interpreting and Verifying EPAS™ Results Based on Raw Ct Values



E. Raw Ct values can be reviewed to assess and confirm EPAS™ results
   i. VIC Ct values for all sample except for sample 601 are between 24.0 – 26.5 and thus are reported as IC Status – PASS.
   ii. VIC Ct values for sample 601 < 24.0 and thus are reported as IC Status – FAIL (overloaded rection)

iii. FAM Ct values for samples 588, 596, 598, and 600 are below the analytical cut-off (≤ 38.0) for KRAS 12/13 (CRCdx® Reaction 1) and are also the lowest FAM Ct values for all other reactions tested and thus are considered KRAS 12/13 mutant positive.
iv. The FAM Ct value for sample 594 is below the analytical cut-off (≤ 38.5) for NRAS 61 (CRCdx® Reaction 7) and are also the lowest FAM Ct values for all other reactions tested and thus are considered NRAS 61 mutant positive.
v. The VIC Ct value for sample 601 is below the acceptance criteria for the assay (< 24.0) for all 8 CRCdx reactions and thus the FAM Ct values are not interpretable. No mutation status result can be given for sample 601. The sample must be re-run with less DNA to bring the VIC value into range of the CRCdx® assay.

15. EPAS™ Error Codes and Resolutions

15.1. EPAS™ Error Codes

Table 7. EPAS™ Error Codes and Resolutions

Code	Type	Window Title	Prompt	Description	Resolution
MSG01	Error	Error Code - MSG01	This username does not exist.	Occurs when a user attempts to login with an incorrect username.	User will need to try to login again with another username.
MSG02	Error	Error Code - MSG02	Invalid username or password. Please ensure the following criteria are met: Username - 5 to 16 alphanumeric characters (underscores and dashes allowed). Password - 8 to 32 alphanumeric characters, with at least 1 uppercase, 1 lowercase, and 1 numeric character.	Occurs when the password or username do not meet criteria when a user account is created or edited.	User will need to enter a new username or password that conforms to the displayed criteria.
MSG03	Confirmation	Confirmation Code - MSG03	The current session will expire in 5 minutes. Do you want to continue the current session?	Occurs when no user input has been recorded by the EPAS application for 10 or more minutes.	User must click Yes to continue the current EPAS session or No to terminate the current session. If no user input is registered, EPAS will terminate the current session after 5 minutes have elapsed.

Code	Type	Window Title	Prompt	Description	Resolution
MSG05	Error	Error Code - MSG05	Invalid data file, invalid assay configuration file, or invalid lot entry.	Occurs when the user attempts to analyze a data file that does not have the expected CRCdx data structure OR when the assay configuration file is corrupted OR when the lot number contains too many characters.	User must ensure the data file is valid, all configuration files are installed and valid, and that the data entered for the Lot Number is less than 30 characters.
MSG06	Error	Error Code - MSG06	Data cannot be analyzed. Please ensure all configuration files are installed.	Occurs when an assay or instrument configuration file is missing and when a data file is loaded into the Parameters Page.	User must reinstall EPAS to recover valid configuration files.
MSG08	Error	Error Code - MSG08	Invalid instrument or assay configuration file detected: [Display List].	Occurs when a configuration (EDN) file has been tampered with or is corrupted. Displays at program start-up and when the Analyze Button is clicked. [Display List] is function that displays a list of corrupted files.	User must reinstall EPAS to recover valid configuration files.

Code	Type	Window Title	Prompt	Description	Resolution
MSG12	Error	Error Code - MSG12	Too many failed login attempts. This account has been disabled, please try again in [Minutes] minutes.	Occurs when a user has failed too many login attempts. The account will be temporarily prevented from logging in for 60 minutes after the last failed attempt. [Minutes] is a function that return the amount of time remaining until the account is re-activated by EPAS.	User must wait until the temporary inactivation period has elapsed. User will have 1 more chance to login with the correct password. Alternatively, an admin level user can log in and reactivate and / or change the password for the disabled account before the temporary account inactivation period has expired.
MSG13	Error	Error Code - MSG13	This account has been inactivated indefinitely. Please ask your administrator to reactive this account.	Occurs when a user account fails to login after the 60-minute temporary inactivation period expires. User will be unable to login.	Admin level user must login and reactive / re-enable the account. Admin can reset password for specified user.
MSG14	Error	Error Code - MSG14	This account is inactive.	Occurs when a user account that has been inactivated attempts to login.	Admin level user must login and re-active / re-enable the specified user account.
MSG15	Error	Error Code - MSG15	You have entered the wrong password, you have [Attempts] more attempt(s).	Occurs when a user enters the wrong password. [Attempts] is a function that displays the number of remaining login attempts before account is temporarily inactivated.	User has 4 total attempts to login with the correct password.

Code	Type	Window Title	Prompt	Description	Resolution
MSG19	Error	Error Code - MSG19	This username already exists. Please choose a different username.	Occurs when a user account is created or edited with a username that already exists in the EPAS database.	User must choose a different username that is not already stored in the EPAS database.
MSG20	Error	Error Code - MSG20	Please select a user account to edit.	Occurs when the "Edit User Account" Button is clicked without a selected user account.	User must select a user account to edit from the Users Table before clicking the Edit User Button.
MSG21	Error	Error Code - MSG21	No data file has been selected for analysis. Please ensure a data file has been loaded through the Data Imports Page by either double-clicking the data file or right-clicking on the data file and selecting Go To Parameters.	Occurs when a data file has not been properly loaded and when the Analyze Button in the Parameters Page or when the Generate Report Button in the Analysis Page is clicked.	User must navigate back to the Data Imports Page and either double-click on a data file or right-click and select Go To Parameters.
MSG22	Error	Error Code - MSG22	Database is currently in use. Close other instances of this application and try again.	Occurs when a user attempts to login to a second instance of EPAS while the first instances of EPAS is running.	Ensure only one instance of EPAS is running. Close all other instances of EPAS or restart computer to clear memory cache.
MSG23	Error	Error Code - MSG23	A user logged into an active session cannot be deleted. Please select another user account.	Occurs when a user attempts to delete the user account currently logged into the active session of EPAS.	User will need to select another user account for deletion.

Code	Type	Window Title	Prompt	Description	Resolution
MSG24	Confirmation	Confirmation Code - MSG24	Deleting user confirmation: Warning, this action cannot be reversed. Are you sure you want to delete this user account: [Username]?	Occurs when a user attempts to delete another user account. Where [Username] is a function that displays the selected username.	Message warns the user that an irreversible action is about to occur. User must click Yes to confirm their choice to delete the selected user account or No to prevent the user account from being deleted and return to the Users Management Tab.
MSG25	Confirmation	Confirmation Code - MSG25	You have unsaved changes. Do you want to discard these changes?	Occurs when the Cancel Button is clicked after changes have been made to an existing user account.	User must click Yes to discard the unsaved changes or No to retain the unsaved changes and remain in the User Details Section.
MSG26	Confirmation	Confirmation Code - MSG26	Entered Data Review Instrument ID: [Value] Lot Number: [Value] Performed By: [Value] Please confirm if the entered data is correct?	Occurs when the Analyze button is clicked. [Value] is a function that saves the value entered in the text field and populates it in the test report.	User must confirm the entered data is correct by clicking Yes to continue or No to return to the Parameters Page.
MSG27	Confirmation	Confirmation Code - MSG27	Deleting data file confirmation: Warning, this action cannot be reversed. Are you sure you want to delete data file: [File Name]?	Occurs when a data file is selected to be deleted. Where [File Name] is a function that displays the selected data file's name.	User must confirm their choice to delete the selected data file by clicking Yes or No to retain the file and return to the Data Imports Page.

Code	Type	Window Title	Prompt	Description	Resolution
MSG28	Error	Error Code - MSG28	Invalid Data File: [Path File Name] The selected data file does not conform to CRCdx data file requirements. Please select another data file for import.	Occurs when a data file is imported that does not contain valid CRCdx data file requirements. [Path File Name] is a function that displays the selected data file's path and file name.	User must select a different data file to import that contains valid CRCdx data file requirements.
MSG29	Error	Error Code - MSG29	Invalid Data File: [Path File Name] The selected data file is corrupted. Please select another data file for import.	Occurs when a data file is imported that does not contain a valid IVD tag. [Path File Name] is a function that displays the selected data file's path and file name.	User must select a different data file to import that was run in the IVD mode of the QSDx instrument.
MSG30	Error	Error Code – MSG30	Control Sample selection error. Please ensure unique samples have been selected for the PC and NTC options.	Shall occur when a user has selected the same sample to be the PC and NTC control reference.	User must select a unique sample for the PC and NTC control selections.

Note: If EPAS™ is being unresponsive to user inputs, restart the program. If the issue persists, ensure the computer configuration meets specifications and attempt to re-install the application. Contact EntroGen Customer (see Section 18 for contact information) if further assistance is required.

15.2. EPAS™ Cybersecurity Measures

EPAS™ software contains several cybersecurity measures to ensure secure operation, protect patient data, and prevent security threats. Unauthorized access or changes to the software will block EPAS™ operation and an error code describing the affected component will be displayed. Please contact EntroGen Technical Support for additional guidance at entrogen.com/support/register.

15.3. QuantStudio™ Dx Instrument safety and EMC standards

The instrument design and manufacture complies with the standards and requirements for safety and electromagnetic compatibility as noted in the QuantStudio™ Dx Real-Time PCR Instrument User Guide, Document ID: 100065652A, Appendix H.

16. CRCdx® Performance Characteristics

16.1. Acceptance Criteria

Table 8. Result Interpretations Performed by EPAS™

Mutation Result	Interpretation
POSITIVE	• Positive and Negative Controls must PASS acceptance criteria. • Sample IC status must PASS acceptance criteria. • Specific mutations identified (FAM Ct ≤ Analytical Cutoff for any CRCdx® reaction).
NEGATIVE	• Positive and Negative Controls must PASS acceptance criteria. • Sample IC status must PASS acceptance criteria. • NO specific mutations identified (FAM Ct > Analytical Cutoff for all CRCdx® reactions).
INVALID	• If either or both PC and NTC do not pass acceptance criteria, the run is considered failed and all results for all samples tested are invalid. • If sample IC status is FAIL (does not meet acceptance criteria), the mutation status cannot be determined. Retest sample with more or less DNA.

Table 9. CRCdx® RAS Analytical Cutoffs for Mutation Amplicon Mutation Calls

Reaction Number	Reaction ID	Target (FAM) Ct Analytical Cutoff	Internal Control (VIC) Ct
1	KRAS 12/13	≤ 38.0	24.0-26.5
2	KRAS 12	≤ 38.0	24.0-26.5
3	KRAS 59/61	≤ 38.5	24.0-26.5
4	KRAS 117	≤ 38.5	24.0-26.5
5	KRAS 146	≤ 38.5	24.0-26.5
6	NRAS 12/13/117	≤ 38.5	24.0-26.5
7	NRAS 61	≤ 38.5	24.0-26.5
8	NRAS 59/146	≤ 38.5	24.0-26.5
--	Negative	> 38.0 (R1 & R2) >38.5 (R3 – R8)	24.0-26.5
--	Loading error	--	<24.0 or >26.5

16.2. Summary of Non-Clinical Studies

16.2.1. Limit of Blank

To assess the performance of the CRCdx® assay in the absence of template to ensure a high concentrated blank sample does not produce an analytical signal, five (5) CRC patient FFPE specimens that are wildtype at the KRAS and NRAS target codons were analyzed at the maximum allowable input as indicated by the internal control reference (VIC) Ct of approximately 24. The specimens were extracted by the Promega Maxell® CSC FFPE DNA Kit and KRAS and NRAS genotype was determined by

Illumina's Praxis™ Extended RAS Panel. Three (3) reagent lots were used to evaluate each specimen (n=10, or 20 per specimen per lot) across eight (8) reactions. The results of the study (N=1600) show an overall negative (wildtype at the KRAS and NRAS loci) call concordance rate of 99.6% (95% CI = 99.1 - 99.8).

16.2.2. Limit of Detection

The CRCdx® RAS Mutation Detection Kit evaluates DNA input concentration using an internal control reaction (VIC) Ct to indicate a sufficient load (VIC Ct = 24 - 26.5). LoD was evaluated at the minimum amplifiable copy input (as indicated by a VIC Ct of 26.5). The LoD study was performed in 3 parts: (i) Primary dilution series, (ii) Secondary dilution series, and (iii) Clinical sample confirmation. The LoD was determined by the lowest MAF where concordance was ≥95% for cell line or surrogate synthetic specimens AND ≥95% concordance using clinical CRC FFPE DNA, when available, to confirm the LoD findings. **Primary dilution series** was performed by assessing CRCdx® using cell line genomic DNA blended with DNA from wildtype FFPE CRC tissue for RAS mutations for which cell lines are available. The limit of detection for rare mutations for which cell line DNA is unavailable was evaluated with synthetic DNA blended with DNA from wildtype FFPE CRC tissue. Twenty-one (21) targets were available in cell line format and fourteen (14) are represented by synthetic surrogates. All samples are diluted to the target MAF in a background of wildtype CRC FFPE DNA. To normalize the study, all targets were assessed at MAFs 50%, 25%, 12.5%, 6.25%, and 3.125% with 20 replicates at each level across two CRCdx® device lots. The percentage of the replicates that were called "Positive" per acceptance criteria was reported for each target dilution as "% correct call." **Secondary dilution series** was performed by assessing CRCdx® on any targets that resulted in an LoD greater than 6.25%. Each target is resolved in three (3) titration levels evenly distributed between the lowest detected MAF and the highest undetected MAF with 20 replicates at each level across two CRCdx® device lots. Four (4) targets required further resolution – KRAS A146T, KRAS A146V, NRAS G12S, and NRAS A59T. Twenty (20) replicates at each level across two CRCdx® device lots was assessed. The percentage of the replicates that were called "Positive" per acceptance criteria was reported for each target dilution as "% correct call". **Clinical sample confirmation** performed once the LoD was determined by the dilution series for all thirty-five (35) targets, on a representative archived CRC clinical FFPE sample for each target per availability. From a pool of >600 CRC FFPE blocks, specimens representing sixteen (16) targets met the requirements for the LoD study. MAF was determined for the extracted analyte using an NGS method for RAS mutations and blended with WT DNA from CRC FFPE tissue to the determined LoD and analyzed at 20 replicates across two CRCdx® device lots. The percentage of the replicates that were called "Positive" per acceptance criteria was reported for each target dilution as "% correct call".

Table 10. Limit of Detection for CRCdx® RAS Mutation Detection Kit Targets

Mutation Target	Reported LoD (%)	Percent prevalence[5] (%)
KRAS G12A	3.13	1.92, 1.93
KRAS G12D	3.13	13.3, 12.83
KRAS G12R	3.13	0.42, 0.4
KRAS G12V	6.25	10.24, 8.92
KRAS G13D	3.13	7.71, 7.33
KRAS G12C	6.25	4.06, 3.22
KRAS G12S	3.13	1.65, 1.67
KRAS Q61H CAT	6.25	1.26, 0.95
KRAS Q61H CAC	3.13	1.26, 0.95
KRAS Q61L	3.13	0.35, 0.35
KRAS Q61R	3.13	0.3, 0.3
KRAS A59E	3.13	No data, No data
KRAS A59G	3.13	No data, No data
KRAS A59T	6.25	No data, 0.2
KRAS K117N AAC	6.25	0.45, 0.46
KRAS K117N AAT	3.13	0.45, 0.46
KRAS K117R	3.13	No data, No data
KRAS K117E	3.13	No data, No data
KRAS A146T	12.5	2.06, 2.57
KRAS A146P	3.13	0.09, 0.09
KRAS A146V	7.81	0.59, 0.58
NRAS G12D	3.13	0.8, 0.79
NRAS G12C	6.25	0.21, 0.21
NRAS G12S	12.5	No data, No data
NRAS G13R	3.13	0.27, 0.27
NRAS G13V	3.13	No data, No data
NRAS K117R	6.25	No data, No data
NRAS Q61H CAC	3.13	0.24, 0.24
NRAS Q61H CAT	3.13	0.24, 0.24
NRAS Q61L	3.13	0.32, 0.32
NRAS Q61K	3.13	0.86, 0.87
NRAS Q61R	3.13	0.6, 0.68
NRAS A146T	3.13	No data, No data
NRAS A59D	3.13	No data, No data
NRAS A59T	7.81	No data, No data

[5]*Colorectal Carcinoma, Colorectal Adenocarcinoma; www.mycancergenome.org*

16.2.3. Input Range

The CRCdx® RAS Mutation Detection Kit evaluates DNA input concentration using an internal control reaction (VIC) Ct to indicate a sufficient load (VIC Ct = 24 - 26.5). The recommended starting input for the CRCdx® RAS Mutation Detection Kit is 24 ng per amplification reaction to target the VIC Ct range. DNA fragmentation and quantification errors may result in various amplifiable DNA input amounts. Nineteen (19) single mutant CRC FFPE specimens and sixteen (16) synthetic gene block blended with wildtype CRC FFPE specimens (all 35 mutant targets represented) were analyzed at six (6) different inputs: 48, 24, 12, 6, 3, and 1.5 ng per amplification reaction. Each sample dilution was analyzed in

duplicate (2) by two (2) reagent lots (n=24 per sample). Internal control linearity (correlation coefficient) across all dilutions for each sample range was 0.99-1.00 and reaction efficiency range was 87.2% – 104.3% demonstrating validity throughout the mass input range. Input loads at 48 ng (n=140), 24 ng (n=140), 12 ng (n=140), and 6 ng (n=140) resulted in a 100.0% correct call rate. 139/140 (99.3%) were called correctly at 3 ng input and 136/140 (97.1%) were called correctly at 1.5 ng input. All 5 missed calls were outside of the acceptable VIC range (VIC Ct = 28.11 – 29.41) and would be flagged by the analysis software (EPAS™) to be re-run at a higher input load.

16.2.4. Minimum Tumor Content

Data generated for the Minimum Tumor Content Study analysis was acquired from the Accuracy and Correlation Study. 391 archived CRC patient FFPE specimens with tumor content ranging from 18-100% were evaluated by CRCdx® and Illumina's Praxis™. Nineteen (19) of the 35 targets are represented in the study. Samples were differentiated into: at or near 20% (18-25%) and >25% tumor content. CRCdx® reported a concordance rate (OPA) of 100.0% (8/8) for samples at or near 20% tumor content of the total samples of 391 with those >25% tumor content yielded 97.9% concordance rate (375/383).

16.2.5. Cross Reactivity

Thirty-five (35) synthetic gene block blended with wildtype CRC FFPE DNA specimens were analyzed by three (3) CRCdx® reagent lots in duplicate. Each sample was loaded near the highest acceptable amplifiable DNA input (VIC = 24.0) to best capture cross-reactive events. FAM Ct values were evaluated based on the assay's acceptance criteria. Two instances of cross reactivity were identified: KRAS G12D showed signal in Reaction 1 (targeted) and Reaction 2 (non-specific) in 2/6 replicates and appeared to be lot specific. KRAS G12S resulted in signal in both Reaction 2 (targeted) and Reaction 1 (non-specific) 6/6 replicates. In all cross-reactive events, the targeted reaction was the earliest Ct. EntroGen's PCR Analysis Software (EPAS™) tool accounts for any cross-reactivity and reports only a single mutation. No additional cross reactivity was observed.

16.2.6. Interference

Necrotic Tissue

Ten (10) wildtype and thirteen (13) KRAS or NRAS mutant CRC FFPE specimens that contained 30% - 70% necrotic tissue, as determined by a board-certified pathologist, were tested. The specimens were extracted by the Promega Maxell® CSC FFPE DNA Kit and KRAS and NRAS genotype was determined by Illumina's Praxis™ Extended RAS Panel. Each specimen was analyzed by two (2) CRCdx® device lots (N=46). The positive percent agreement was 100% (26/26) and negative percent agreement was 100% (20/20) for all observations. The data supports that up to 70% necrotic tissue content does not interfere with the call results for the CRCdx® RAS Mutation Detection Kit.

Exogenous Substances

Potentially interfering substances present in the manual processing steps of DNA extraction process (Promega Maxell® CSC FFPE DNA Kit) were tested at 10-fold excess concentration (or the greatest excess concentration allowed by volume of the extraction tube) during the extraction process. Six (6) substances were tested and compared to the untreated control. Eight (8) wildtype and eight (8) KRAS or NRAS mutant CRC FFPE specimens were extracted by two (2) DNA extraction kit lots under each of

seven (7) conditions and analyzed by two (2) CRCdx® device lots (n=32 per condition). The specimens were genotyped by Illumina's Praxis™ Extended RAS Panel. CRCdx® correctly called 98.4% (188/191) of the treated specimens. Three (3) samples – one extracted with excess paraffin, one extracted with excess blue dye, and one extracted with excess RNase A – were called incorrectly. One (1) sample extracted with excess lysis buffer did not yield sufficient DNA to be tested and was excluded from the study. The results demonstrate that the exogenous reagents tested do not interfere with the call results for the CRCdx® RAS Mutation Detection Kit.

16.2.7. Number of Curls

Two (2), four (4), and eight (8) curls from three (3) previously characterized CRC patient FFPE specimens and (3) FFPE cell lines (Horizon Discovery) were extracted using Promega's Maxwell® CSC DNA FFPE Kit. The samples were quantified using Thermo Fisher Scientific Qubit™ dsDNA BR Assay Kit and diluted to 4 ng/ul. Each extract was assessed using one (1) CRCdx® reagent lot at 24 ng input in duplicate. The results of the study (N=288) show that overall percent mutation agreement for both specimen types within all curl counts was 100% (95% CI = 95.4% - 100.0%).

16.2.8. DNA Extraction Method Characterization

Data generated for this DNA Extraction method study was acquired from the Limit of Detection and Accuracy and Correlation (in part) Studies performed for CRCdx, in which thirty-five (35) CRC FFPE extraction were performed on nineteen (19) CRC FFPE blocks each representing a total of the nineteen (19) targeted mutations and analyzed with CRCdx® assay. Twenty-two (22) Maxwell® CSC FFPE DNA kit lots and nine (9) CRCdx® device lots were used during the studies. All samples were previously characterized by Illumina's Praxis Extended RAS NGS Panel. LoD samples were generated by diluting extracted CRC FFPE DNA to the previously determined LoD in a background of wildtype CRC FFPE DNA. Sixteen (16) samples each representing a different mutation targeted by CRCdx® were analyzed by two (2) CRCdx® device lots across twenty (20) replicates (n=317). Accuracy samples were extracted by Promega's Maxwell® CSC DNA FFPE kit and analyzed within the analytical VIC range (n=383). All DNA samples yield >95% Positive percent agreement (PPA = 99.8%), Negative Percent Agreement (NPA = 95.2%), and Overall Percent Agreement (OPA = 98.7%) for all replicates across both studies (N=710) for CRCdx® performances. The results validate Promega's Maxwell® CSC DNA FFPE kit (Promega Cat#AS1350) to yield quality DNA suitable for downstream CRCdx® RAS Mutation Detection analysis.

16.2.9. Cross Contamination

A checkerboard patterned plate where wells containing high positive samples and NTC wells were alternated was used for this study. Eight replicates (8) of five (5) samples, two (2) CRC FFPE specimens, two (2) cell lines, and one (1) synthetic gene block in a background of wildtype CRC FFPA DNA, with high MAFs that ranged from 45.1% - 70.6% were analyzed by three (3) operators on non-consecutive days in two (2) checkerboard plate formats (N=480). A full plate of NTCs was also included. All test wells (100%) displayed FAM and VIC signals that met the acceptance criteria demonstrating that there was no significant cross-contamination

from neighboring wells containing high %MAF mutant specimens. The study results show that the manual processing steps are not vulnerable to cross contamination events resulting from well-to-well carryover by means of 100% correct call by three (3) different operators.

16.2.10. Precision

The Precision of the CRCdx® RAS Mutation Detection Kit was assessed using the same batch of sixteen (16) mutant CRC FFPE specimens, seven (7) mutant cell lines, and twelve (12) synthetic mutant gene block in a background of wildtype CRC FFPE DNA samples, and two (2) wildtype CRC FFPE specimens throughout the study. Each mutant sample was diluted to 1.5X LoD or 3X LoD and ran in duplicate (2) across three (3) CRCdx® device lots by two (2) trained operators at each of the three (3) external sites (N=2592 initially planned samples). Each CRC FFPE specimen was extracted using Promega's Maxwell® CSC FFPE DNA Extraction Kit by EntroGen and %MAF was determined using an NGS method for RAS mutations. Samples were diluted to the target %MAF in a background of wildtype CRC FFPE DNA and shipped frozen. CROs stored reagents and samples at -15° to -20°C and used within one week of thawing. A Positive Control (PC) and Non-Template Control (NTC) was included in each run to qualify each plate. The run files and exported line data generated by the external sites were sent to EntroGen for Ct data analysis and mutation analysis using EPAS™. Per the study design, both replicates of a sample on the same plate run were required to pass. Overall, the study had a first pass rate of 90.0% (1166/1296 mutant and wildtype duplicates). 2.7% (35/1296) sample data were not accepted due to invalid positive control results, 3.5% (46/1296) sample data were not accepted due to invalid NTC results, and 49/1296 (3.8%) sample results were not accepted due to user error. Samples with unacceptable data results during first pass were subsequently re-run to complete the study. A total of 2571 first-pass and re-run sample replicates, 2499 mutant and 72 wildtype, were included in the data analysis. Correct call rate across all samples, dilutions, replicates, lots, users, sites, instruments, and days was 100.0% (2499/2499 mutant and 72/72 wildtype samples).

Table 11. Overall Agreement Correct Calls Per Site

Site	CRCdx® Reaction	Correct Wildtype Call/Total Wildtype Samples	Correct Mutant Call/Total Mutant Samples	Percent Agreement (%)
1	1	24/24	118/118	100.0
	2		47/47	100.0
	3		162/162	100.0
	4		95/95	100.0
	5		72/72	100.0
	6		144/144	100.0
	7		120/120	100.0
	8		68/68	100.0
2	1	24/24	120/120	100.0
	2		48/48	100.0
	3		168/168	100.0
	4		96/96	100.0
	5		71/71	100.0
	6		144/144	100.0
	7		120/120	100.0
	8		70/70	100.0
3	1	24/24	120/120	100.0
	2		48/48	100.0
	3		168/168	100.0
	4		96/96	100.0
	5		68/68	100.0
	6		144/144	100.0
	7		120/120	100.0
	8		72/72	100.0
Total		**72/72**	**2499/2499**	**100.0**

Precision was determined by assessing the within-lot, within-run, within user/day, between-lot, between-run, and between user/day variability (%CV). Variability was evaluated using the reported FAM Ct values (excludes wildtype because no passing FAM Ct values were produced). The within-lot variability was 0.35% - 3.37% (N=2499 mutant samples). The within-run variability was 0.09% - 1.53% (N=2499 mutant samples). The within-user/day variability was 0.11% - 4.54% (N=2499 mutant samples). The between-user variability was 0.19% - 2.15% (N=2499 mutant samples). The between lot variability was 0.30% - 3.34% (N=2499 mutant samples). The between-CRO variability was 0.35% to 3.37% (N=2499 mutant samples).

All of the precision measures in this study are with a %CV of less than 5.0% (%CV < 5.0%). The Repeatability Study results demonstrate strong agreement and acceptable precision-repeatability of the measurements (consistency in calling and Ct results) made for Within-Site/ Lab, Within-Run, and Between-Site/ Lab with respect to user-to-user, run-to-run, lot-to-lot, day-to-day, instrument-to-instrument, and site-to-site variabilities for CRCdx® RAS assay (N=2499 mutant samples and N=72 wildtype samples).

Reproducibility was determined by assessing the between-site and within-site variability (%CV). The Ct data dispersion results show a %CV < 5.0% across all sites (%CV = 0.30% - 3.34%) for an overall average %CV of 1.35% (N=2499 mutant samples). These results demonstrate strong agreement and reliability for reproducibility of the measurements made with respect to user-to-user, run-to-run, lot-to-lot, day-to-day, instrument-to-instrument, and site-to-site variabilities for CRCdx® RAS assay.

Table 12. Per-Site and Overall Ct Variability

Site	Number of Observations (n)	Percent Coefficient of Variation (%CV)	
		Mean	Mean
1	827	1.04%	0.30% - 3.21%
2	836	1.18%	0.36% - 3.04%
3	836	1.29%	0.37% - 3.34%
All	2499	1.35%	0.58% - 2.89%

The Percent Correct Calls (PCCs) by variant and corresponding 95% two-sided confidence intervals was calculated using the Wilson score method. The Percent Correct Call (PCC) across all mutant samples was 100% (lower bound 95% CI: 99.84%) when excluding samples with failed sample QC.

Table 13. Reproducibility Percent Correct Call by Variant

Gene	Exon	Amino Acid Change	Nucleotide Change	N	Percent Correct Call (%)	95% CI
KRAS	2	G12A	c.35G>C	72	72/72 (100.0)	(94.9,100.0)
		G12D	c.35G>A	72	72/72 (100.0)	(94.9,100.0)
		G12R	c.34G>C	72	72/72 (100.0)	(94.9,100.0)
		G12V	c.35G>T	71	71/71 (100.0)	(94.9,100.0)
		G13D	c.38G>A	71	71/71 (100.0)	(94.9,100.0)
		G12C	c.34G>T	72	72/72 (100.0)	(94.9,100.0)
		G12S	c.34G>A	71	71/71 (100.0)	(94.9,100.0)
		KRAS Exon 2 Total		501	501/501 (100.0)	(99.2,100.0)
	3	Q61H (CAT)	c.183A>T	70	70/70 (100.0)	(94.8,100.0)
		Q61H (CAC)	c.183A>C	68	68/38 (100.0)	(94.7,100.0)
		Q61L	c.182A>T	72	72/72 (100.0)	(94.9,100.0)
		Q61R	c.182A>G	72	72/72 (100.0)	(94.9,100.0)
		A59E	c.176C>A	72	72/72 (100.0)	(94.9,100.0)
		A59G	c.176C>G	72	72/72 (100.0)	(94.9,100.0)
		A59T	c.175G>A	72	72/72 (100.0)	(94.9,100.0)
		KRAS Exon 3 Total		498	498/498 (100.0)	(99.2,100.0)
	4	K117N (AAC)	c.351A>C	71	71/71 (100.0)	(94.9,100.0)
		K117N (AAT)	c.351A>T	72	72/72 (100.0)	(94.9,100.0)
		K117R	c.350A>G	72	72/72 (100.0)	(94.9,100.0)
		K117E	c.349A>G	72	72/72 (100.0)	(94.9,100.0)
		A146T	c.436G>A	72	72/72 (100.0)	(94.9,100.0)
		A146P	c.436G>C	72	72/72 (100.0)	(94.9,100.0)
		A146V	c.437C>T	67	67/67 (100.0)	(94.6,100.0)
		KRAS Exon 4 Total		498	498/498 (100.0)	(99.2,100.0)

Gene	Exon	Amino Acid Change	Nucleotide Change	N	Percent Correct Call (%)	95% CI
	All KRAS Exons			**1497**	**1497/1497 (100.0)**	**(99.7,100.0)**
NRAS	6	G12D	c.35G>A	72	72/72 (100.0)	(94.9,100.0)
		G12C	c.34G>T	72	72/72 (100.0)	(94.9,100.0)
		G12S	c.34G>A	72	72/72 (100.0)	(94.9,100.0)
		G13R	c.37G>C	72	72/72 (100.0)	(94.9,100.0)
		G13V	c.38G>T	72	72/72 (100.0)	(94.9,100.0)
		NRAS Exon 2 Total		360	360/360 (100.0)	(98.94,100.0)
	7	Q61H (CAC)	c.183A>C	72	72/72 (100.0)	(94.9,100.0)
		Q61H (CAT)	c.183A>T	72	72/72 (100.0)	(94.9,100.0)
		Q61L	c.182A>T	72	72/72 (100.0)	(94.9,100.0)
		Q61K	c.181C>A	72	72/72 (100.0)	(94.9,100.0)
		Q61R	c.182A>G	72	72/72 (100.0)	(94.9,100.0)
		A59D	c.176C>A	70	70/70 (100.0)	(94.8,100.0)
		A59T	c.175G>A	72	72/72 (100.0)	(94.9,100.0)
		NRAS Exon 3 Total		502	502/502 (100.0)	(99.2,100.0)
	8	A146T	c.436G>A	68	68/68 (100.0)	(94.7,100.0)
		K117R	c.350A>G	72	72/72 (100.0)	(94.9,100.0)
		NRAS Exon 4 Total		140	140/140 (100.0)	(97.3,100.0)
	All NRAS Exons Total			**1002**	**1002/1002 (100.0)**	**(99.0,100.0)**
All KRAS and NRAS Exons Total				**2499**	**2499/2499 (100.0)**	**(99.8,100.0)**

16.2.11. Stability Studies

16.2.11.1. Extracted DNA from FFPE Specimens

i. Frozen DNA Extract Stability (-15˚C to -20˚C): Eight (8) KRAS or NRAS mutant containing CRC FFPE specimens, and one (1) wildtype CRC FFPE specimen was extracted by two (2) extraction kit lots and stored at -15˚C to -25˚C for the indicated duration. Seven of the Eight CRCdx® reactions are represented due to the rarity of some reaction targets (KRAS G13D, G12C, Q61H CAC, Q61H CAT, K117N AAT, A146T, and NRAS G12D, Q61K). Five (5) replicates per sample were analyzed by two (2) CRCdx® device lots at six (6) timepoints: 0, 7, 14, 21, 28, and 35 days. The results of the study (FAM N=475; VIC N=477) showed 100% correct call and no statistically significant mean difference of Ct measurand was observed. DNA extracts prepared by the Promega Maxwell® CSC FFPE DNA Extraction Kit are stable for up to 28 days when stored at -15˚C to -25˚C.

ii. Refrigerated DNA Extract Stability (2˚C to 8˚C): Eight (8) KRAS or NRAS mutant containing CRC FFPE specimens, and one (1) wildtype CRC FFPE specimen was extracted by two (2) extraction kit lots and stored at 2˚C to 8˚C for the indicated duration. Five of the Eight CRCdx® reactions are represented due to the rarity of some reaction targets samples (KRAS G12V, G12A, G12S, G12C, Q61H CAT, Q61H CAC, A146T, and NRAS G12D). Three (3) replicates per sample were analyzed by three (3) CRCdx® device lots at five (5) timepoints: 0, 3, 5, 7, and 9 days. The results of the study (FAM N=351; VIC N=357) showed 100% correct call and no statistically significant mean difference of Ct measurand was

observed. DNA extracts prepared by the Promega Maxwell® CSC FFPE DNA Extraction Kit are stable for up to 7 days when stored at 2˚C to 8˚C.

iii. Freeze-Thaw Cycling DNA Extract Stability: Eight (8) KRAS or NRAS mutant containing CRC FFPE specimens, and one (1) wildtype CRC FFPE specimen was extracted by two (2) extraction kit lots and placed at -15˚C to -25˚C prior to the starting the study. Each sample was thawed at room temperature (22˚C to 28˚C) at the indicated timepoint and placed back in the freezer (-15˚C to -25˚C) for the subsequent timepoint. Five of the Eight CRCdx® reactions are represented due to the rarity of some reaction targets samples (KRAS G12V, G12A, G12S, G12C, Q61H CAT, Q61H CAC, A146T, and NRAS G12D). Three (3) replicates per sample were analyzed by three (3) CRCdx® device lots at four (4) timepoints: 0, 1, 2, and 3 weeks (1 cycle per week). The results of the study (FAM N=279; VIC N=286) showed 100% correct call and no statistically significant mean difference of Ct measurand was observed. DNA extracts prepared by the Promega Maxwell® CSC FFPE DNA Extraction Kit are stable following a maximum of three (3) freeze-thaw cycles.

16.2.11.2. Open Vial Stability

Three (3) lots of reagents were used to test seven (7) KRAS or NRAS mutant CRC FFPE specimens, one (1) NRAS mutant cell line, and one (1) wildtype CRC FFPE specimen. CRC FFPE specimens were extracted and all mutant samples (KRAS G12A, G12C, Q61L, K117N AAT, A146V, and NRAS G12D, Q61L, A146T) were diluted to 3X and 9X LoD in wildtype CRC FFPE DNA background to be analyzed at a low DNA input to best capture false calls. Each sample dilution was run in triplicate per timepoint. All reagents for the study were thawed at room temperature (18˚C – 26˚C) and stored in the refrigerator (2˚ to 8˚C) until the indicated timepoint, 0, 1, and 4 weeks. Reaction plates were assembled with master mixes and DNA and ran immediately. The results of the study (FAM N=433; VIC N=420) showed 100% correct call and no statistically significant mean difference of Ct measurand was observed. CRCdx® RAS Mutation Detection Kit reagents can be stored in the refrigerator (2˚ to 8˚C), as necessary, for up to 3 weeks without compromising the performance characteristics of the assay.

16.2.11.3. Closed Vial Stability

Three (3) lots of reagents were used to test six (6) KRAS and NRAS mutant cell lines, two (2) synthetic surrogate NRAS mutant samples, and one (1) wildtype CRC FFPE specimen. Contrived samples were used for this Closed Vial stability study because of the lack of sufficient availability and reliability of CRC patient FFPE specimens, a consequence from the length of the study. All mutant samples (KRAS G12D, G12S, Q61H CAT, K117N AAT, A146P, and NRAS G13V, Q61R, A146T) were diluted to 3X and 9X LoD in wildtype CRC FFPE DNA background to be analyzed at a low DNA input to best capture false calls. Each sample dilution was run in triplicate per timepoint. All reagents for the study were stored at -15˚C to -25˚C and thawed at room temperature (18˚C – 26˚C) for the indicated timepoints, 0, 1, 2, 3, 4, 8, and 12 months. Reaction plates were assembled with master mixes and DNA and ran immediately. The results of the study (FAM N=988; VIC N=983) showed 100% correct call and no statistically significant trend of the measurement drifts was observed. CRCdx® RAS Mutation Detection Kit reagents can be stored frozen (-15˚ to -25˚C) for up 10 months.

16.2.11.4. Freeze Thaw Stability

Three (3) lots of reagents were used to test seven (7) KRAS or NRAS mutant CRC FFPE specimens, one (1) NRAS mutant cell line, and one (1) wildtype CRC FFPE specimen. CRC FFPE specimens were extracted and all mutant samples (KRAS G12A, G12S, Q61L, K117N AAT, A146P, and NRAS G12D, Q61L, A146T) were diluted to 3X and 9X LoD in wildtype CRC FFPE DNA background to be analyzed at a low DNA input to best capture false calls. Each sample dilution was run in triplicate per timepoint. CRCdx® reagents were stored at -15˚C to -25˚C between uses. Reagents were removed from the freezer and thawed at room temperature (18˚C – 26˚C) at each timepoint, 0, 1, 2, 3, 4, and 5 weeks, and returned to the freezer after use for the subsequent timepoints. Reaction plates were assembled with master mixes and DNA and ran immediately. The results of the study (FAM N=827; VIC N=869) showed 100% correct call and no statistically significant trend of the measurement drifts was observed. CRCdx® RAS Mutation Detection Kit reagents can be freeze thawed as necessary for up to five (5) cycles with good laboratory practice (light exposure prevention, controlled temperature, etc.) without compromising the performance characteristics of the assay

16.2.11.5. Master Mix Stability

Three (3) lots of reagents were used to test seven (7) KRAS or NRAS mutant CRC FFPE specimens, one (1) NRAS mutant cell line, and one (1) wildtype CRC FFPE specimen. CRC FFPE specimens were extracted and all mutant samples (KRAS G12A, G12S, Q61L, K117N AAT, A146P, and NRAS G12D, Q61L, A146T) were diluted to 3X and 9X LoD in wildtype CRC FFPE DNA background to be analyzed at a low DNA input to best capture false calls. Each sample dilution was run in triplicate per timepoint. Master mixes were prepared by mixing the appropriate amount of Mutation Detection Reaction Mix, Primer/Probe Mix, and Water. Master mixes were tested at four (4) incubation timepoints, 0, 30, 60, and 120 minutes, and at two (2) dark storage conditions (room temperature: 18˚C – 26˚C; or refrigerated: 2˚C – 8˚C). Following incubation, master mixes were aliquoted into 96-well reaction plates and DNA samples added just prior to the start of amplification. The results of the study (FAM N=1126; VIC N=1140) showed 100% correct call and no statistically significant trend of the measurement drifts was observed. CRCdx® master mixes can be stored in the dark at either room temperature (18˚C – 26˚C) or refrigerated (2˚C – 8˚C) for up to 100 minutes without compromising the performance characteristics of the CRCdx® assay.

16.2.11.6. Plate Stability

Three (3) lots of reagents were used to test seven (7) KRAS or NRAS mutant CRC FFPE specimens, one (1) NRAS mutant cell line, and one (1) wildtype CRC FFPE specimen. CRC FFPE specimens were extracted and all mutant samples (KRAS G12A, G12S, Q61L, K117N AAT, A146P, and NRAS G12D, Q61L, A146T) were diluted to 3X and 9X LoD in wildtype CRC FFPE DNA background to be analyzed at a low DNA input to best capture false calls. Each sample dilution was run in triplicate per timepoint. Master mixes were prepared by mixing the appropriate amount of Mutation Detection Reaction Mix, Primer/Probe Mix, and Water and aliquoted into 96-well reaction plates. DNA samples were added to the appropriate master mixes and the assembled plates were sealed and stored in the dark at either room temperature (18˚C – 26˚C) or refrigerated (2˚C – 8˚C) for 0, 2, or 6 hours. The results of the study (FAM N=845; VIC N=838) showed 100% correct call and no statistically significant

trend of the measurement drifts was observed. CRCdx® assembled reaction plates can be stored in the dark at either room temperature (18°C – 26°C) or refrigerated (2°C – 8°C) for up to 5 hours without compromising the performance characteristics of the CRCdx® assay.

16.2.11.7. Transportation Simulation

Three (3) lots of reagents were used to test seven (7) KRAS or NRAS mutant CRC FFPE specimens, one (1) NRAS mutant cell line, and one (1) wildtype CRC FFPE specimen. CRC FFPE specimens were extracted and all mutant samples (KRAS G12A, G12S, Q61L, K117N AAT, A146P, and NRAS G12D, Q61L, A146T) were diluted to 3X and 9X LoD in wildtype CRC FFPE DNA background to be analyzed at a low DNA input to best capture false calls. Each sample dilution was run in triplicate per timepoint. The reagents were subjected to one of the following four (4) treatments: No treatment control (-15° to -25°C); Refrigerated Treatment (2° to 8°C) for 24 hours; Room Temperature Treatment (18° to 26°C) for 24 hours; and Extreme Heat Treatment (32 °to 42°C) for 24 hours. Reagents were placed in the freezer for a minimum of 24 hours (-15° to -25°C) following treatment. Reagents were removed from the freezer and thawed at room temperature (18°C – 26°C) and reaction plates were assembled with master mixes and DNA and run immediately. The results of the study (FAM N=573; VIC N=576) showed 100% correct call and no statistically significant mean difference of Ct measurand was observed.

16.3. Summary of Analytical and Clinical Accuracy Studies

16.3.1. Accuracy and Correlation Studies to an approved FDA Test - Praxis™

Accuracy of CRCdx® RAS was demonstrated via external concordance analysis to estimate percent agreement with the FDA-approved NGS-method Praxis™. Additional performance analysis of CRCdx® was inferred using the Non-Inferiority (NI) statistical model. A total of 662 archived CRC patient FFPE specimens were acquired from commercial biorepositories. Of the 662 specimens, 398 (60.1%) met the requirements to be screened and provided successful sequencing results by the comparator companion diagnostic (CCD) Praxis™ Panel.

16.3.2. Concordance Rates

Agreement between CRCdx® RAS (FCD) and Illumina's Praxis™ (CCD) were assessed at the specimen level (i.e., mutation detected vs. mutation not detected) in accordance with the intended use – i.e., a subject will not receive the companion therapeutic if any actionable KRAS or NRAS mutation is detected.

- The PPA was defined as the proportion of positive specimens as detected by CRCdx® assay among all positive specimens as determined by the Praxis™ Extended RAS NGS Panel.
- The NPA was defined as the proportion of negative specimens as detected by CRCdx® assay among all negative specimens as determined by the Praxis™ Extended RAS NGS Panel.
- The OPA was defined as the overall proportion of concordant specimens as detected by CRCdx® assay among all specimens as determined by the Praxis™ Extended RAS NGS Panel.

The PPA is 99.6% with lower bounds of the 95% CI at 97.5%; NPA is 92.0% with lower bound of 95% CI at 86.8% (Table 3). Fourteen (14) samples were called wildtype by Praxis™ and mutant by CRCdx® RAS.

The low NPA results may be explained by the disparity in sensitivities between the two analytical platforms. There were no invalids reported by CRCdx® RAS. The data does not include 264 specimens that failed to qualify for Praxis™.

Table 134. Summary of Positive, Negative, and Overall Percent Agreement for CRCdx® with Praxis™

		Praxis			PPA	NPA	OPA
		Mutant	WT	Total			
CRCdx	Mutant	223	14	237	99.6%	92.0%	96.2%
	WT	1	160	161			
	Total	224	174	398			
				95% CI	97.5% to 100.0%	86.8% to 95.5%	93.9% to 97.9%

The Positive and Negative Percent Agreement by variant compared to Praxis™ is below.

Table 15. Accuracy and Correlation PPA and NPA Results by Variant Compared to Praxis™

Gene	CRCdx® Reaction	Amino Acid Change	Nucleotide Change	TP	FN	FP	TN	Total	PPA (95% CI*)	NPA (95% CI*)
KRAS	1	G12A	c.35G>C	10	0	1	387	398	100.0 (69.2-100.0)	99.74 (98.5-100.0)
		G12D	c.35G>A	72	0	2	324	398	100.0 (95.0-100.0)	99.39 (97.2-100.0)
		G12R	c.34G>C	2	0	0	396	398	100.0 (15.8-100.0)	100.0 (99.1-100.0)
		G12V	c.35G>T	37	0	0	361	398	100.0 (90.5-100.0)	100.0 (99.0-100.0)
		G13D	c.38G>A	34	0	1	363	398	100.0 (89.7-100.0)	99.73 (98.1-100.0)
		All Reaction 1 Mutations		155	0	11†	232	398	100.0 (97.7-100.0)	95.47 (91.4-97.12)
	2	G12C	c.34G>T	11	0	0	387	398	100.0 (71.5-100.0)	100.0 (99.1-100.0)
		G12S	c.34G>A	16	0	0	382	398	100.0 (79.4-100.0)	100.0 (99.0-100.0)
	3	Q61H (CAT)	c.183A>T	2	0	0	396	398	100.0 (15.8-100.0)	100.0 (99.1-100.0)
		Q61H (CAC)	c.183A>C	3	0	0	395	398	100.0 (29.2-100.0)	100.0 (99.1-100.0)
		Q61L	c.182A>T	1	0	0	397	398	100.0 (25.0-100.0)	100.0 (99.1-100.0)
		Q61R	c.182A>G	0	0	0	398	398	NE	100.0 (99.1-100.0)
		A59E	c.176C>A	0	0	0	398	398	NE	100.0 (99.1-100.0)
		A59G	c.176C>G	0	0	0	398	398	NE	100.0 (99.1-100.0)
		A59T	c.175G>A	0	0	1	397	398	NE	99.75 (98.5-100.0)
	4	K117N (AAC)	c.351A>C	0	0	0	398	398	NE	100.0 (99.1-100.0)
		K117N (AAT)	c.351A>T	1	0	0	397	398	100.0 (25.0-100.0)	100.0 (99.1-100.0)
		K117R	c.350A>G	0	0	0	398	398	NE	100.0 (99.1-100.0)
		K117E	c.349A>G	0	0	0	398	398	NE	100.0 (99.1-100.0)
	5	A146T	c.436G>A	12	1	0	385	398	92.31 (53.9-100.0)	100.0 (99.1-100.0)
		A146P	c.436G>C	2	0	0	396	398	100.0 (15.8-100.0)	100.0 (99.1-100.0)
		A146V	c.437C>T	6	0	0	392	398	100.0 (54.1-100.0)	100.0 (99.1-100.0)
NRAS	6	G12D	c.35G>A	4	0	0	394	398	100.0 (39.8-100.0)	100.0 (99.1-100.0)
		G12C	c.34G>T	0	0	0	398	398	NE	100.0 (99.1-100.0)
		G12S	c.34G>A	0	0	0	398	398	NE	100.0 (99.1-100.0)
		G13R	c.37G>C	2	0	0	396	398	100.0 (15.8-100.0)	100.0 (99.1-100.0)
		G13V	c.38G>T	0	0	0	398	398	NE	100.0 (99.1-100.0)
		K117R	c.350A>G	0	0	0	398	398	NE	100.0 (99.1-100.0)
		All Reaction 6 Mutations		6	0	2†	390	396	100.0 (54.1-100.0)	99.49 (97.7-100.0)
	7	Q61H (CAC)	c.183A>C	0	0	0	398	398	NE	100.0 (99.1-100.0)
		Q61H (CAT)	c.183A>T	0	0	0	398	398	NE	100.0 (99.1-100.0)
		Q61L	c.182A>T	3	0	0	395	398	100.0 (29.2-100.0)	100.0 (99.1-100.0)
		Q61K	c.181C>A	3	0	0	395	398	100.0 (29.2-100.0)	100.0 (99.1-100.0)
		Q61R	c.182A>G	2	0	0	396	398	100.0 (15.8-100.0)	100.0 (99.1-100.0)

Gene	CRCdx® Reaction	Amino Acid Change	Nucleotide Change	TP	FN	FP	TN	Total	PPA (95% CI*)	NPA (95% CI*)
	8	A146T	c.436G>A	0	0	0	398	398	NE	100.0 (99.1-100.0)
		A59D	c.176C>A	0	0	0	398	398	NE	100.0 (99.1-100.0)
		A59T	c.175G>A	0	0	0	398	398	NE	100.0 (99.1-100.0)
Total				223	1	14	13701	13930	99.55 (96.0-100.0)	99.90 (99.8-100.0)

NE = not estimable; NA=not applicable

†CRCdx does not differentiate between mutations in each reaction. False positives that were not resolved are accounted for in the 'All reaction' FP column

*For all agreements (PPA, NPA) not equal to 100%, bootstrap method was used to calculate the two-sided 95% confidence intervals (CIs); For all agreements equal to 100%, exact method was used to calculate the 95% CIs. Boot strap confidence intervals are obtained from bootstrap sampling of subjects (maintaining all mutation results nested within subject) with replacement 9,999 times (1)

16.3.3. Analytical Concordance

To further evaluate the analytical accuracy and understand NPA results, the original ACCOR study included comparisons between CRCdx®, Praxis, and two validated NGS panels on a subset of the available samples (N=151). Of the 398 samples, EntroGen was additionally able to assess accuracy by testing 151 samples using two validated NGS panels excluding 247 samples. 107 samples were exhausted, 63 samples were not concentrated enough (qPCR quantification), 35 failed library preparation (qPCR library quantification or melt curve analysis), and 42 failed to produce sequencing results. The 77 wildtype samples and 74 KRAS/NRAS mutant samples were screened by (Praxis, CRCdx, and validated NGS for RAS mutations. Out of 151 samples tested using two validated NGS tests, the resulting concordance with aggregated results from two orthogonal NGS methods was: PPA = 96.3%, with 95% CI 89.7% to 99.2%, and NPA 92.8%, with 95% CI 83.9% to 97.6%.

Table 16. Overall Concordance Between CRCdx and Validated NGS Sample Population Subset

		LDT-NGS			PPA	NPA
		Mutant	WT	Total		
CRCdx	Mutant	79	5	84	96.3%	92.8%
	WT	3	64	67		
	Total	82	69	151		

Out of 117 samples tested using validated NGS test 1, the resulting concordance was: PPA = 100.0%, with 95% CI 94.0% to 100.0%, and NPA 93.0%, with 95% CI 83.0% to 98.1%.

Out of 37 samples tested using validated NGS test 2, the resulting concordance was: PPA = 92.0%, with 95% CI 74.0% to 99.0%, and NPA 91.7 %, with 95% CI 61.5% to 99.8%.

Using the data generated with the two validated NGS assays and comparative analysis with a composite method from all three orthogonal comparator methods used, EntroGen investigated 15 samples that had discordant results with Praxis, as shown in Table below. Of the 15 discordant samples, three (3) did not have validated NGS assay results, and therefore no composite orthogonal result one (1) sample with Praxis mutation positive was CRCDx positive but the mutation determined by Praxis is not one of the targeted mutations in CRCDx, therefore it was considered discordant; two (2) samples with Praxis mutation negative result were CRCDx positive, five (5) were classified as apparent negative status when assessed using composite orthogonal method, six (6) were classified as apparent positive status when assessed using composite orthogonal method, and one (1) sample with Praxis mutation positive

but CRCDx mutation negative result was classified as apparent mutation positive. The agreement calculated using results for 151 samples tested with the composite orthogonal comparator was: PPA = 98.7%, with 95% CI 93.1% to 100%, and NPA 93.1%, with 95% CI 84.5% to 97.7%.

Table 17. Results for Discordant Sample Results Between Praxis RAS and CRCdx Compared to Composite Orthogonal Result

Praxis Call	CRCdx Call	Validated NGS Result	Status Classification
WT	KRAS 12/13	Low %MAF KRAS G12D (< 5.0%)	Apparent Positive
WT	KRAS 12/13	Low %MAF KRAS G12D (< 5.0%)	Apparent Positive
WT	KRAS 12/13	Low %MAF KRAS G13D (< 5.0%)	Apparent Positive
WT	KRAS 12/13	Low %MAF KRAS G12A (< 5.0%)	Apparent Positive
WT	KRAS 12/13	High %MAF KRAS G13D (> 5.0%)	Apparent Positive
WT	KRAS 59/61	High %MAF KRAS Q61L (> 5.0%)	Apparent Positive
KRAS A146T	WT	KRAS A146T 6.2% MAF	Apparent Positive
WT	KRAS 12/13	No evidence of positive mutation	Apparent Negative
WT	KRAS 12/13	No evidence of positive mutation	Apparent Negative
WT	KRAS 12/13	No evidence of positive mutation	Apparent Negative
WT	KRAS 12/13	No evidence of positive mutation	Apparent Negative
WT	NRAS 12/13/117	No evidence of positive mutation	Apparent Negative

16.3.4. Non-Inferiority

The Non-Inferiority Hypothesis Testing via external concordance demonstrated that CRCdx® was Non-Inferior to Praxis™ by comparing CRCdx® to Replicated Praxis™ results.

To assess whether the agreement between FCD (CRCdx®) and CCD (Praxis Extended RAS) is similar to the agreement between two replicates of CCD (denoted CCD1 as replicate 1 and CCD2 as replicate 2). The approach assumes replicates of CCD hypothetically yield the same results for CCD (i.e., CCD1=CCD2).

Four (4) total hypotheses were tested from the concordance data between CRCdx® and Praxis™ results.

1. **Hypothesis 1**: Tests if the difference observed (PPAC1C2 minus PPAC1F) is greater than or equal to a minimum difference of 10%. **PPAC1C2** represents **PPA between CCD1 and CCD2**. **PPAC1F** represents **PPA with CCD1 for FCD**.
2. **Hypothesis 2:** Tests if the difference observed (PPAC2C1 minus PPAC2F) is greater than or equal to a minimum difference of 10%. **PPAC2C1** represents **PPA between CCD2 and CCD1**. **PPAC2F** represents **Positive Percent Agreement with CCD2 for FCD**.
3. **Hypothesis 3:** Tests if the difference observed (NPAC1C2 minus NPAC1F) is greater than or equal to a minimum difference of 10%. **NPAC1C2** represents **NPA between CCD1 and CCD2. NPAC1F** represents **NPA with CCD1 for FCD.**
4. **Hypothesis 4:** Tests if the difference observed (NPAC2C1 minus NPAC2F) is greater than or equal to a minimum difference of 10%. **NPAC2C1** represents **NPA between CCD2 and CCD1**. **NPAC2F** represents **NPA with CCD2 for FCD.**

The parameter estimates of percent agreement differences between the devices were below the acceptable non-inferiority margin (10%). The upper bounds of the confidence intervals for the two NPA-related hypothesis tests were > 10% likely due to the disparity in sensitivities between the different analytical platforms. The results demonstrated that performance of the follow-on companion diagnostic CRCdx® will be non-inferior to the comparator companion diagnostic Praxis™ in a clinical setting.

Table 16. Non-Inferiority Hypothesis Testing

	Null Hypothesis	Alternate Hypothesis	Calculated NI	Lower 95% CI	Upper 95% CI
Hypothesis 1	PPAC1C2 - PPAC1F ≥ 10%	PPAC1C2 - PPAC1F < 10%	4.464%	0	1.364%
Hypothesis 2	PPAC2C1 − PPAC2F ≥ 10%	PPAC2C1 − PPAC2F < 10%	4.464%	0	1.389%
Hypothesis 3	NPAC1C2 − NPAC1F ≥ 10%	NPAC1C2 − NPAC1F < 10%	8.046%	4.469%	11.732%
Hypothesis 4	NPAC2C1 − NPAC2F ≥ 10%	NPAC2C1 − NPAC2F < 10%	8.046%	3.468%	10.309%

17. Revision History

For notification about any change in the IFU, please register to EntroGen technical support at entrogen.com/support/register.

Last Change: August 2023	
Change	**Affected section(s)**
Initial commercial version.	All
Updated EPAS™ error codes	15

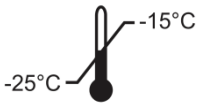
18. Appendix

Symbols

Catalog Number

Lot/Batch Number

Expiration Date



Storage Conditions

Manufactured by

Intended Use

Number of tests per kit

Consult instructions for use

Prescription Use Only

Contact Information

For technical assistance and information about this product, please visit support.entrogen.com or call us at 818-716-1070.

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