cobas® KRAS Mutation Test

FOR IN VITRO DIAGNOSTIC USE.

cobas® KRAS Mutation Test	**KRAS**	24 Tests	M/N: 05852170190

Refer to the **cobas**® DNA Sample Preparation Kit (M/N 05985536190) for sample preparation information.

INTENDED USE

The **cobas**® KRAS Mutation Test, for use with the **cobas**® 4800 System, is a real-time PCR test for the detection of somatic mutations in codons 12, 13 and 61 of the KRAS gene in DNA derived from formalin-fixed paraffin-embedded human colorectal cancer (CRC) tumor tissue. The test is intended to be used as an aid in the identification of CRC patients who should not be treated with Erbitux® (cetuximab) or with Vectibix® (panitumumab) when KRAS Codon 12 or 13 mutation is detected. Safety and efficacy of Erbitux® (cetuximab) or Vectibix® (panitumumab) have not been established in patients whose tumors have Codon 61 mutation.

Specimens are processed using the **cobas**® DNA Sample Preparation Kit for manual sample preparation and the **cobas**® z 480 analyzer for automated amplification and detection. The **cobas**® KRAS Mutation Test detects the following KRAS mutations.

Codon	Mutation ID	AA Change	COSMIC ID
12	c.34G>T	12C	516
	c.34G>A	12S	517
	c.34G>C	12R	518
	c.35G>T	12V	520
	c.35G>A	12D	521
	c.35G>C	12A	522
13	c.38G>A	13D	532
61	c.181C>A	61K	549
	c.181C>G	61E	550
	c.182A>C	61P	551
	c.182A>G	61R	552
	c.182A>T	61L	553
	c.183A>C	61Hc	554
	c.183A>T	61Ht	555

SUMMARY AND EXPLANATION OF THE TEST

The KRAS protein is a member of the superfamily of small G proteins. KRAS acts as a GDP/GTP-regulated switch to convey extracellular signals that influence cell proliferation, apoptosis and remodeling of the actin cytoskeleton. Mutations affecting amino acids 12 or 13, which occur in a variety of human malignancies, including colorectal cancer (CRC), lock the enzyme in the GTP-bound, activated form, resulting in constitutive signaling and thereby contributing to the oncogenic process.[1]

KRAS mutations are observed in 24%-43% of colorectal tumors.[2-3] Although over 3000 point mutations of the KRAS gene have been identified, most occur in codons 12 or 13 (~82% in codon 12 and ~17% in codon 13).[4] KRAS mutations in other codons (e.g., codon 61) are less common (2-3% of mutations) and while infrequent, they have been shown to result in constitutive activation of KRAS just as in codon 12 and 13 mutations.[4]

Cetuximab and panitumumab are monoclonal antibodies which target the epidermal growth factor receptor (EGFR) and are approved for use in patients with metastatic colorectal cancer. Although 50% to 80% of colorectal tumors overexpress EGFR, EGFR protein expression and gene amplification have only limited predictive value in determining the likelihood of response to cetuximab or panitumumab.[5]

However, there is now strong evidence to show that the presence of KRAS mutations correlates with lack of response to EGFR-targeted antibody therapy in patients with metastatic colorectal cancer and that, in some situations, the use of EGFR-targeted antibody therapy in this patient subgroup may be detrimental.[6-7] The supporting evidence for these findings comes from:

- Retrospective analyses of single-arm studies[8-9]
- Retrospective analyses of randomized studies[10-11]
- Prospective randomized studies[12]

As a consequence of these and other studies[13-14], full KRAS and NRAS mutation testing is recommended for the selection of patients to receive anti-EGFR antibody therapy by major oncology organizations in the US (ASCO, NCCN)[15-16] and Europe (ESMO).[17] Furthermore, US and European regulatory authorities have restricted the use of these agents to patients with KRAS wild-type tumors.[18-19]

The **cobas**® KRAS Mutation Test is a PCR-based assay designed to identify the presence of somatic mutations involving codons 12, 13 and 61 of the proto-oncogene KRAS. Based on data in the COSMIC database (2015 v72), the mutations detected by the **cobas**® KRAS Mutation Test account for >97% of all reported KRAS mutations in CRC patients. Thus, the test is intended to be used as an aid in the identification of CRC patients for whom treatment with Erbitux® (cetuximab) or with Vectibix® (panitumumab) may be indicated based on a no mutation detected result.

PRINCIPLES OF THE PROCEDURE

The **cobas**® KRAS Mutation Test (**cobas** KRAS Test) is based on two major processes: (1) manual specimen preparation to obtain genomic DNA from formalin-fixed, paraffin-embedded tissue (FFPET); and (2) PCR amplification of target DNA using complementary primer pairs and an oligonucleotide probe labeled with fluorescent dye. One probe is designed to detect the KRAS codon 12/13 sequence in exon 2, and the other probe is designed to detect the KRAS codon 61 sequence in exon 3 of the KRAS gene. Mutation detection is achieved by melting curve analysis by the **cobas**® z 480 analyzer. A mutant control, negative control, and calibrator are included in each run to confirm the validity of the run.

Specimen Preparation

FFPET specimens are processed and genomic DNA isolated using the **cobas**® DNA Sample Preparation Kit, a generic manual specimen preparation based on nucleic acid binding to glass fibers. A deparaffinized 5-µm section of an FFPET specimen is lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released genomic DNA from DNases. Subsequently, isopropanol is added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the genomic DNA is bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and then eluted with an aqueous solution. The amount of genomic DNA is spectrophotometrically determined and adjusted to a fixed concentration to be added to the amplification/detection mixture. The target DNA is then amplified and detected on the **cobas**® z 480 analyzer using the amplification and detection reagents provided in the **cobas** KRAS Test kit.

PCR Amplification

Target Selection

The **cobas** KRAS Test kit uses primers that define an 85 base-pair sequence for exon 2 containing KRAS codons 12 and 13 and a 75 base-pair sequence for exon 3 containing KRAS codon 61 in human genomic DNA. Amplification occurs only in the regions of the KRAS gene between the primers; the entire KRAS gene is not amplified.

Target Amplification

A derivative of *Thermus* species Z05 DNA polymerase is utilized for target amplification. First, the PCR reaction mixture is heated to denature the genomic DNA and expose the primer target sequences. As the mixture cools, the upstream and downstream primers anneal to the target DNA sequences. The Z05 DNA polymerase, in the presence of a divalent metal ion and excess dNTPs, extends each annealed primer, thus synthesizing a second DNA strand. This completes the first cycle of PCR, yielding a double-stranded DNA copy which includes the targeted 85 base-pair and 75 base pair regions of the KRAS gene. This process is repeated for a number of cycles, with each cycle effectively doubling the amount of amplicon DNA.

Automated Real-time Mutation Detection

The **cobas**® z 480 analyzer is capable of measuring, in real-time, the amount of fluorescence generated by specific PCR products. After amplification, each amplicon generated using the **cobas** KRAS Test is subjected to a melting program in which the temperature is ramped from 40°C to 95°C (TaqMelt). The wild-type specific probe is bound to both wild-type and mutant amplicon at low temperatures. In the bound state, the fluorescein reporter dye on the 5' end of the probe is sufficiently far away from the 3' end quencher dye, allowing the fluorescent dye to emit a specific wave length of light. As the temperature rises, the probe dissociates from the amplicon, allowing the quencher dye to come into close proximity to the fluorescent dye, decreasing the amount of measurable fluorescence. Amplicons with a perfect match to the probe (wild-type) melt at a higher temperature than amplicons with one or more mismatches (mutant). The amount of fluorescence at each temperature increment is measured and the melting temperature(s) are calculated. The presence of a mutant KRAS sequence in exon 2, codons 12 and 13 and in exon 3, codon 61 can be detected when the melting temperatures are within specified ranges. To avoid detection of codon 12 and codon 13 silent mutations (no amino acid change), a modified base serves as a universal base and produces a melting temperature within the wild-type range.

Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the **cobas** KRAS Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP).[20] The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing thymidine. Deoxyuridine is not present in naturally occurring DNA but is always present in amplicon due to the use of dUTP in place of thymidine triphosphate as one of the nucleotide triphosphates in the Reaction Mix reagent; therefore, only amplicons contain deoxyuridine. Deoxyuridine renders contaminating amplicons susceptible to destruction by AmpErase enzyme prior to amplification of the target DNA. The AmpErase enzyme, which is included in the Reaction Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step at alkaline pH, the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA non-amplifiable. The AmpErase enzyme is inactive at temperatures above 55ºC, i.e., throughout the thermal cycling steps, and therefore does not destroy the target amplicon.

REAGENTS

cobas® KRAS Mutation Test (KRAS)
24 Tests (M/N: 05852170190)

Kit components	Reagent ingredients	Quantity per kit	Safety symbol and warning[a]
KRAS MIX **(KRAS Reaction Mix)**	Tricine buffer Potassium acetate Potassium hydroxide Glycerol 4.76% Dimethyl sulfoxide <0.9% dNTPs <0.1% Z05 DNA polymerase (microbial) <0.1% AmpErase (uracil-N-glycosylase) enzyme (microbial)	4 x 0.3 mL	 **WARNING** H317: May cause an allergic skin reaction. H412: Harmful to aquatic life with long lasting effects. P261: Avoid breathing mist or vapours. P273: Avoid release to the environment. P280: Wear protective gloves. P333 + P313: If skin irritation or rash occurs: Get medical advice/ attention. P362 + P364: Take off contaminated clothing and wash it before reuse. P501: Dispose of contents/ container to an approved waste disposal plant. 55965-84-9 reaction mass of 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (3:1)
MGAC **(Magnesium acetate)**	Magnesium acetate 0.09% Sodium azide	4 x 0.2 mL	N/A
KRAS OM1 **(KRAS Oligo Mix 1)**	Tris-HCl buffer EDTA Poly-rA RNA (synthetic) 0.1% ProClin® 300 preservative[b] <0.01% Upstream and downstream KRAS Primers <0.01% Fluorescent labeled KRAS probe	2 x 0.3 mL	 **WARNING** H317: May cause an allergic skin reaction. H412: Harmful to aquatic life with long lasting effects. P261: Avoid breathing mist or vapours. P273: Avoid release to the environment. P280: Wear protective gloves. P333 + P313: If skin irritation or rash occurs: Get medical advice/ attention. P362 + P364: Take off contaminated clothing and wash it before reuse. P501: Dispose of contents/ container to an approved waste disposal plant. 55965-84-9 reaction mass of 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (3:1)

cobas® KRAS Mutation Test (KRAS)
24 Tests (M/N: 05852170190)

Kit components	Reagent ingredients	Quantity per kit	Safety symbol and warning[a]
KRAS OM2 **(KRAS Oligo Mix 2)**	Tris-HCl buffer EDTA Poly-rA RNA (synthetic) 0.1% ProClin® 300 preservative[b] <0.01% Upstream and downstream KRAS Primers <0.01% Fluorescent labeled KRAS probe	2 x 0.3 mL	 **WARNING** H317: May cause an allergic skin reaction. H412: Harmful to aquatic life with long lasting effects. P261: Avoid breathing mist or vapours. P273: Avoid release to the environment. P280: Wear protective gloves. P333 + P313: If skin irritation or rash occurs: Get medical advice/ attention. P362 + P364: Take off contaminated clothing and wash it before reuse. P501: Dispose of contents/ container to an approved waste disposal plant. 55965-84-9 reaction mass of 5-chloro-2-methyl-2H-isothiazol-3-one and 2-methyl-2H-isothiazol-3-one (3:1)
KRAS MC **(KRAS Mutant Control)**	Tris-HCl buffer EDTA Poly-rA RNA (synthetic) 0.05% Sodium azide <0.001% plasmid DNA containing KRAS exon 2 and 3 sequences (microbial) <0.001% KRAS wild-type DNA (cell culture)	4 x 0.1 mL	N/A
KRAS CAL **(KRAS Calibrator)**	Tris-HCl buffer EDTA Poly-rA RNA (synthetic) 0.05% Sodium azide <0.001% KRAS wild-type DNA (cell culture)	4 x 0.1 mL	N/A
DNA SD **(DNA Specimen Diluent)**	Tris-HCl buffer 0.09% Sodium azide	2 x 3.5 mL	N/A

[a] Product safety labeling primarily follows EU GHS guidance
[b] Hazardous substance

WARNINGS AND PRECAUTIONS

A. **FOR IN VITRO DIAGNOSTIC USE.**

B. This test is for use with formalin-fixed paraffin-embedded colorectal cancer tissue specimens.

C. Do not pipette by mouth.

D. Do not eat, drink or smoke in laboratory work areas.

E. Avoid microbial and DNA contamination of reagents.

F. Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

G. Do not use kits after their expiration dates.

H. Do not pool reagents from different kits or lots.

I. Safety Data Sheets (SDS) are available on request from your local Roche office.

J. Gloves must be worn and must be changed between handling specimens and reagents to prevent contamination.

K. To avoid contamination of the working Master Mix (working MMX) with DNA specimens, Amplification and Detection should be performed in an area separated from DNA Isolation. The amplification and detection work area should be thoroughly cleaned before working MMX preparation. For proper cleaning, all surfaces including racks and pipettors should be thoroughly wiped with 0.5% sodium hypochlorite* solution followed by wiping with a 70% ethanol solution.

Note:** **Commercial liquid household bleach typically contains sodium hypochlorite at a concentration of 5.25%. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.

L. Specimens should be handled as infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories[21] and in the CLSI Document M29-A3.[22]

M. **MGAC**, **KRAS MC, KRAS CAL,** and **DNA SD** contain sodium azide. Sodium azide may react with lead and copper plumbing to form highly explosive metal azides. While disposing of sodium azide containing solutions down laboratory sinks, flush the drains with a large volume of cold water to prevent azide buildup.

N. Wear eye protection, 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.

O. All disposable items are for one time use. Do not reuse.

P. Do not use disposable items beyond their expiration date.

Q. Do not use sodium hypochlorite solution (bleach) for cleaning the **cobas**® z 480 analyzer. Clean the **cobas**® z 480 analyzer according to procedures described in the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance.

R. For additional warnings, precautions and procedures to reduce the risk of contamination for the **cobas**® z 480 analyzer, consult the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance.

S. The use of sterile disposable pipettes and DNase-free pipette tips is recommended.

STORAGE AND HANDLING REQUIREMENTS

A. Store **KRAS MIX**, **MGAC**, **KRAS OM1, KRAS OM2, KRAS MC, KRAS CAL**, and **DNA SD** at -25ºC to -15°C. Once opened, these reagents are stable for 4 uses over 60 days or until the expiration date, whichever comes first.

B. Allow all reagents to thaw at 15ºC to 30ºC for at least 1 hour prior to use. Once thawed, use the reagents within 1 hour, and return any unused reagent to -25ºC to -15°C storage within 1 hour. Once opened, each reagent vial, except **DNA SD**, may be used for pipetting up to 4 aliquots over 60 days or until the expiration date, whichever comes first.

C. **KRAS OM1, KRAS OM2,** and working MMX (prepared by the addition of **KRAS OM1 or KRAS OM2** and **MGAC** to **KRAS MIX**) should be protected from prolonged exposure to light.

D. Once prepared, working MMX must be stored at 2ºC to 8ºC in the dark. The prepared specimens and controls must be added within 1 hour of preparation of the working MMX.

E. Processed specimens (extracted DNA) are stable for up to 24 hours at 15ºC to 30°C or up to 14 days at 2ºC to 8°C or up to 60 days at -15ºC to -25°C or after undergoing 3 freeze thaws when stored at -15ºC to -25°C. Extracted DNA should be amplified within the recommended storage periods or before the expiration date of the **cobas**® DNA Sample Preparation Kit used to extract the DNA, whichever comes first.

F. Amplification must be started within 1 hour from the time that the processed specimens and controls are added to the working MMX (prepared by the addition of **KRAS OM1** or **KRAS OM2** and **MGAC** to **KRAS MIX**).

MATERIALS REQUIRED BUT NOT PROVIDED

- **cobas**® DNA Sample Preparation Kit (Roche M/N 05985536190)
- **cobas**® 4800 System AD-plate and Sealing Film (Roche M/N 05232724001)
- **cobas**® 4800 Sealing Film Applicator (Roche M/N 04900383001)
- Adjustable Pipettors* (capacity 10 µL, 20 µL, 200 µL, and 1000 µL) with aerosol barrier or positive displacement DNase-free tips
- Locking-lid microcentrifuge tubes (1.5-mL sterile, RNase/DNase free, PCR grade) (Any vendor)
- Spectrophotometer for measuring DNA concentration**
- Vortex mixer**
- Microcentrifuge tube racks
- Disposable gloves, powderless

* *Pipettors should be maintained according to the manufacturer's instructions and accurate within 3% of stated volume. Aerosol barrier or positive displacement DNase-free tips must be used where specified to prevent specimen degradation and cross-contamination.*

** *All equipment should be properly maintained according to manufacturer's instructions.*

Instrumentation and Software

- **cobas**® z 480 analyzer
- **cobas**® 4800 SR2 System Control Unit with OSXP image
- **cobas**® 4800 SR2 System Software Version 2.1 or higher
- KRAS Analysis Package Software Version 1.1.0.1330 or higher
- Barcode Reader (Roche M/N 05339910001)
- Printer HP P2055d (Roche M/N 05704375001)

SPECIMEN COLLECTION, TRANSPORT, AND STORAGE

NOTE: Handle all specimens as if they are capable of transmitting infectious agents.

A. Specimen Collection

Colorectal cancer (CRC) FFPET specimens have been validated for use with the **cobas** KRAS Test.

B. Specimen Transport

FFPET specimens can be transported at 15ºC to 30ºC. Transportation of FFPET specimens must comply with country, federal, state, and local regulations for the transport of etiologic agents.[23]

C. Specimen Storage

FFPET specimens may be stored at 15ºC to 30°C for up to 12 months after the date of tissue collection. Five-µm sections mounted on slides may be stored at 15 to 30°C for up to 60 days.

INSTRUCTIONS FOR USE

NOTE: Only FFPET sections of 5-µm thickness containing at least 10% tumor content by area are to be used with the cobas KRAS Test. Any specimen containing less than 10% tumor content by area should be macro-dissected prior to DNA extraction.

NOTE: Refer to the cobas® 4800 System – Operator's Manual or cobas® 4800 System – User Assistance for detailed operating instructions for the cobas® z 480 analyzer.

Run Size

A single run can include from 1 to 45 specimens (plus controls and calibrator). When running more than 24 specimens, multiple **cobas** KRAS Test kits of the same lot will be required.

The **cobas** KRAS Test contains sufficient reagents for 8 runs of 3 specimens (plus controls and calibrator) for a maximum of 24 specimens per kit.

Workflow

DNA Isolation

DNA is isolated from FFPET specimens using the **cobas**® DNA Sample Preparation Kit (M/N 05985536190).

Macro-dissection

If the sample contains less than 10% tumor content by area, the sample must be macro-dissected as part of the sample preparation.

DNA Quantitation

NOTE: Measurement of DNA concentration should be performed immediately after the DNA Isolation procedure and prior to storage.

A. Mix each DNA Stock by vortexing for 5 seconds.

B. Quantify DNA using a spectrophotometer according to the manufacturer's protocol. Use **DNA EB** from the **cobas**® DNA Sample Preparation Kit as the blank for the instrument. An average of two consistent readings is necessary. The two measurements should be within ±10% of each other when the DNA concentration readings are ≥ 20.0 ng/µL. For DNA concentration readings < 20.0 ng/µL, the two measurements should be within ± 2 ng/µL. If the two measurements are not within +/- 10% of each other when the DNA concentration readings are ≥ 20.0 ng/µL or within +/- 2 ng/µL when the DNA concentration readings are < 20.0 ng/µL, an additional 2 readings must be taken until the requirements are met. The average of these two new measurements should then be calculated.

NOTE: The DNA Stock from the processed negative control (NEG CT) does not need to be measured.

C. The DNA Stock concentration from the specimens must be ≥ 4 ng/µL to perform the **cobas** KRAS Test. Two amplification/detections are run per specimen, using 25 µL of a 2 ng/µL dilution of DNA Stock (total of 50 ng DNA) for each amplification/detection.

NOTE: Each DNA Stock must have a minimum concentration of 4 ng/µL to perform the cobas KRAS Test. If the concentration of a DNA Stock is <4 ng/µL, repeat the deparaffinization, DNA isolation, and DNA quantitation procedures for that specimen using two 5-µm FFPET sections. For mounted specimens, after deparaffinization, combine the tissue from both sections into one tube, immerse the tissue in DNA TLB + PK from the cobas® DNA Sample Preparation Kit, and perform DNA isolation and quantitation. For unmounted specimens, combine two sections into one tube and perform deparaffinization, DNA isolation and quantitation. If the DNA Stock is still <4 ng/µL, then request another FFPET specimen.

NOTE: Processed specimens (extracted DNA) are stable for up to 24 hours at 15°C to 30°C or up to 14 days at 2°C to 8°C or up to 60 days at -15°C to -25°C or after undergoing 3 freeze thaws when stored at -15°C to -25°C. Extracted DNA should be amplified within the recommended storage periods or before the expiration date of the cobas® DNA Sample Preparation Kit used to extract the DNA, whichever comes first.

AMPLIFICATION AND DETECTION

NOTE: To avoid contamination of working MMX with DNA specimens, Amplification and Detection should be performed in an area separated from DNA Isolation. The amplification and detection work area should be thoroughly cleaned before working MMX preparation. For proper cleaning, all surfaces including racks and pipettors should be thoroughly wiped with 0.5% sodium hypochlorite solution followed by wiping with a 70% ethanol solution. Commercial liquid household bleach typically contains sodium hypochlorite at a concentration of 5.25%. A 1:10 dilution of household bleach will produce a 0.5% sodium hypochlorite solution.

Instrument Set-Up

Refer to the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance for detailed instruction for the **cobas**® z 480 set up.

Test Order Set-up

Refer to the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance for detailed instructions on the **cobas** KRAS Test workflow steps.

Dilution Calculation of Specimen DNA Stock

<u>Dilution Calculation for DNA Stock Concentrations from 4 ng/µL to 28 ng/µL</u>

NOTE: DNA stocks from specimens should be diluted immediately prior to amplification and detection.

NOTE: Two (2) amplification/detections are run for each specimen requiring a total volume of 50 µL (25 µL each for MMX1 and for MMX2) of a 2 ng/µL dilution of DNA Stock (total of 100 ng DNA).

A. For each specimen, calculate the volume (µL) of DNA stock needed:

µL of DNA stock = (70 µL x 2 ng/µL) ÷ DNA Stock concentration [ng/µL]

B. For each specimen, calculate the volume (µL) of DNA Specimen Diluent (**DNA SD**) needed:

µL of **DNA SD** = 70 µL – µL of DNA Stock

Example:

DNA stock concentration = 6.5 ng/µL

A. µL of DNA Stock = (70 µL x 2 ng/µL) ÷ 6.5 ng/µL = 21.5 µL

B. µL of **DNA SD** = (70 µL – 21.5 µL) = 48.5 µL

Dilution Calculation for DNA Stock Concentrations >28 ng/µL

NOTE: DNA Stocks from specimens should be diluted immediately prior to amplification and detection.

NOTE: Two (2) amplification/detections are run for each specimen requiring a total volume of 50 µL (25 µL each for MMX1 and for MMX2) of a 2 ng/µL dilution of DNA stock (total of 100 ng DNA).

A. At DNA Stock concentrations > 28 ng/µL, use the following formula to calculate the amount of DNA Specimen Diluent (**DNA SD**) required to prepare at least 70 µL of diluted DNA stock. This is to ensure that each specimen uses a minimum of 5 µL of DNA stock.

B. For each specimen, calculate the volume (µL) of **DNA SD** needed to dilute 5 µL of DNA Stock to 2 ng/µL:

Vol. of **DNA SD** required in µL = [(5 µL of DNA stock x DNA stock concentration in ng/µL) / 2 ng/µL] – 5 µL

Example:

DNA stock concentration = 31.7 ng/µL

A. Vol. of **DNA SD** required in µL = [(5 µL x 31.7 ng/µL) / 2 ng/µL] – 5 µL = 74.3 µL

B. Use the calculated volume of **DNA SD** to dilute 5 µL of DNA stock.

Specimen Dilution

NOTE: Remove the specimen diluent (DNA SD) from -15°C to -25°C storage and thaw at 15°C to 30°C for at least 1 hour before DNA dilution. Vortex each reagent for 5 seconds and collect liquid at the bottom of the tube before use.

A. Prepare the appropriate number of 1.5 mL microcentrifuge tubes for DNA Dilutions by labeling them with the proper specimen identification.

B. Using a pipettor with an aerosol-resistant tip, pipette the calculated volumes of **DNA SD** into the respectively labeled tubes. Pipette 35 µL of **DNA SD** into a tube labeled as **NEG CT**.

C. Vortex each DNA stock and the negative control for 5 to 10 seconds.

D. Using a pipettor with an aerosol-resistant pipette tip (new tip for each pipetting), gently pipette the calculated volume of each DNA stock into the respective tube containing **DNA SD**. Pipette 35 µL of negative control (extracted eluate) into the **NEG CT** tube.

E. Cap the tubes and vortex each for 5 to 10 seconds.

F. Change gloves.

Preparation of Working Master Mixes (MMX 1 and MMX 2)

NOTE: KRAS OM1, KRAS OM2, and working MMX are light-sensitive and must be protected from prolonged exposure to light.

NOTE: Due to the viscosity of the KRAS MIX and working MMX, pipette slowly to ensure all mix is completely dispensed from the tip.

NOTE: The KRAS MIX, KRAS OM1 and KRAS OM2 may appear clear to yellow. This does not affect the performance of the reagent.

Prepare two bulk working MMX, one containing **KRAS OM1** and the other containing **KRAS OM2** in separate 1.5 mL microcentrifuge tubes.

A. Calculate the volume of **KRAS MIX** required for each working MMX using the following formula:

Volume of **KRAS MIX** required = (Number of Specimens + 2 Controls + 1 Calibrator +1) x 10 µL

B. Calculate the volume of **KRAS OM1** or **KRAS OM2** required for each working MMX using the following formula:

Volume of **KRAS OM1 or KRAS OM2** required = (Number of Specimens + 2 Controls + 1 Calibrator +1) x 10 µL

C. Calculate the volume of **MGAC** required for each working MMX using the following formula:

Volume of **MGAC** required = (Number of Specimens + 2 Controls + 1 Calibrator +1) x 6 µL

Use Table 1 to determine the volume of each reagent needed for the preparation of working MMX based on the number of specimens included in the run.

Table 1

Volumes of Reagents Needed for Working MMX 1 and Working MMX 2

Volumes of Reagents Needed for Working MMX											
# of Specimens*		**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
KRAS Mix	**10 µL**	**50**	**60**	**70**	**80**	**90**	**100**	**110**	**120**	**130**	**140**
KRAS OM1 or OM2	**10 µL**	**50**	**60**	**70**	**80**	**90**	**100**	**110**	**120**	**130**	**140**
MGAC	**6 µL**	**30**	**36**	**42**	**48**	**54**	**60**	**66**	**72**	**78**	**84**
Total Volume µL		**130**	**156**	**182**	**208**	**234**	**260**	**286**	**312**	**338**	**364**

* Includes sufficient volumes for 1 tube per specimen, 2 control tubes, 1 calibrator tube, and 1 extra tube.

D. Remove the appropriate number of **KRAS MIX, KRAS OM1, KRAS OM2,** and **MGAC** vials from -25°C to -15°C storage. Allow all reagents to thaw at 15°C to 30°C for at least 1 hour prior to use. Vortex each reagent for 5 seconds and collect liquid at the bottom of the tube before use. Label a sterile microcentrifuge tube for working MMX 1 and working MMX 2.

NOTE: Working MMXs must be prepared within 1 hour once reagents are thawed. Once thawed, return any remaining, unused reagents to -25°C to -15°C storage within 1 hour after use.

E. Add the calculated volume of **KRAS MIX** to the working MMX tubes.

F. Add the calculated volume of **KRAS OM1** or **KRAS OM2** to their respective working MMX tube.

G. Add the calculated volume of **MGAC** to the working MMX tubes.

H. Vortex the tubes for 3 to 5 seconds to ensure adequate mixing.

NOTE: Specimens, controls and calibrator should be added to the AD-plate within 1 hour after the preparation of the working MMXs.

NOTE: Use only cobas® 4800 System AD-plate and Sealing Film (Roche M/N 05232724001).

Figure 1

Sample Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	KRAS MC MMX1	KRAS MC MMX2	Sample 6 MMX1	Sample 6 MMX2	Sample 14 MMX1	Sample 14 MMX2	Sample 22 MMX1	Sample 22 MMX2				
B	KRAS NC MMX1	KRAS NC MMX2	Sample 7 MMX1	Sample 7 MMX2	Sample 15 MMX1	Sample 15 MMX2	Sample 23 MMX1	Sample 23 MMX2				
C	KRAS CAL MMX1	KRAS CAL MMX2	Sample 8 MMX1	Sample 8 MMX2	Sample 16 MMX1	Sample 16 MMX2	Sample 24 MMX1	Sample 24 MMX2				
D	Sample 1 MMX1	Sample 1 MMX2	Sample 9 MMX1	Sample 9 MMX2	Sample 17 MMX1	Sample 17 MMX2						
E	Sample 2 MMX1	Sample 2 MMX2	Sample 10 MMX1	Sample 10 MMX2	Sample 18 MMX1	Sample 18 MMX2						
F	Sample 3 MMX1	Sample 3 MMX2	Sample 11 MMX1	Sample 11 MMX2	Sample 19 MMX1	Sample 19 MMX2						
G	Sample 4 MMX1	Sample 4 MMX2	Sample 12 MMX1	Sample 12 MMX2	Sample 20 MMX1	Sample 20 MMX2						
H	Sample 5 MMX1	Sample 5 MMX2	Sample 13 MMX1	Sample 13 MMX2	Sample 21 MMX1	Sample 21 MMX2						

PCR Set-up

A. Pipette 25 µL of working MMX into each reaction well of the AD-plate that is needed for the run. Do not allow the pipettor tip to touch the plate outside the well.

- Add working MMX1 (containing **KRAS OM1**) to the AD-plate wells in the odd-numbered columns (1, 3, 5, etc.)
- Add working MMX2 (containing **KRAS OM2**) to the AD-plate wells in the even-numbered columns (2, 4, 6, etc.)

B. Pipette 25 µL of **KRAS MC** into wells **A1** and **A2** of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.

C. Using a new pipettor tip, pipette 25 µL of **NEG CT** into wells **B1** and **B2** of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.

D. Using a new pipettor tip, pipette 25 µL of **KRAS CAL** into wells **C1** and **C2** of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.

NOTE: Each run must contain positive control (KRAS MC) in wells A1 and A2, negative control (NEG CT) in wells B1 and B2, and calibrator (KRAS CAL) in wells C1 and C2 or the run will be invalidated.

NOTE: Change gloves as needed to protect against specimen-to-specimen contamination and external PCR reaction tube contamination.

E. Using new pipettor tips for each diluted specimen DNA, add 25 µL of the first specimen DNA to wells **D1** and **D2** of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for the diluted DNA from the second specimen (wells **E1** and **E2**). Follow the template in Figure 1 until all specimens' DNA Dilutions are loaded onto the AD-plate. Ensure that all liquid is collected at the bottom of the wells.

F. Cover the AD-plate with sealing film (supplied with the plates). Use the sealing film applicator to seal the film firmly to the AD-plate.

G. Confirm that all liquid is collected at the bottom of each well before starting PCR.

NOTE: Amplification and Detection should be started within 1 hour after the addition of the first specimen DNA dilution to the working MMX.

Starting PCR

Refer to the **cobas**® 4800 System – Use Assistance for detailed instructions on the **cobas** KRAS Test workflow steps.

INTERPRETATION OF RESULTS

NOTE: All run and specimen validation is performed by the cobas®4800 software.

NOTE: A valid test run may include both valid and invalid sample results.

For a valid run, specimen results are interpreted as shown in Table 2.

Table 2
Result Interpretation of cobas KRAS Test

Test Result	***Mutation Result***	***Interpretation***
Mutation Detected in Codon 12/13	Codon 12/13	Mutation detected in KRAS codon 12/13.
Mutation Detected in Codon 61	Codon 61	Mutation detected in KRAS codon 61. Safety and efficacy of Erbitux® (cetuximab) or Vectibix® (panitumumab) have not been established in patients whose tumors have Codon 61 mutation.
No Mutation Detected*	N/A	Mutation not detected in KRAS codon 12/13 and 61.
Invalid	N/A	Specimen result is invalid. Repeat the testing of specimens with invalid results following the instructions outlined in the "**Retesting of Specimens with Invalid Results**" section below.
Failed	N/A	Failed run due to hardware or software failure. Contact your local Roche office for technical assistance

* *A "No Mutation Detected" result does not preclude the presence of a mutation in the KRAS 12/13 and 61 codon sites because results depend on percent mutant sequences, adequate specimen integrity, absence of inhibitors, and sufficient DNA to be detected.*

Retesting of Specimens with Invalid Results

A. Repeat dilution of the invalid specimen DNA stock starting from "**Dilution Calculation of Specimen DNA Stock**" and "**Specimen Dilution**" procedures in the "**AMPLIFICATION and DETECTION**" section.

B. After performing the DNA stock dilution to 2 ng/µL described in "**Specimen Dilution"** continue with "**Preparation of Working Master Mixes (MMX1 and MMX2)**" and the remainder of the amplification and detection procedure.

NOTE: If the specimen remains invalid after retesting or there was not enough DNA stock to prepare another dilution, obtain a new 5-µm FFPET section of tissue and re-isolate DNA using the cobas DNA Sample Preparation Kit (M/N 05985536190) and repeat testing.

QUALITY CONTROL

One **cobas**® KRAS Test set of Mutant Control (**KRAS MC**), negative control (**NEG CT**) and KRAS Calibrator (**KRAS CAL**) for working MMX1 and working MMX2 is included in each run. A run is valid if the KRAS Mutant Control (**KRAS MC)** wells (**A1** and **A2**), the negative control **(NEG CT)** wells (**B1** and **B2**), and the KRAS Calibrator (**KRAS CAL**) wells (**C1** and **C2**) are valid. If the KRAS Mutant Control (**KRAS MC**), negative control (**NEG CT**) or KRAS Calibrator (**KRAS CAL**) for working MMX1 or working MMX2 are invalid, the entire run is invalid and must be repeated. Prepare a fresh dilution of the previously isolated specimen DNA Stock to set up a new AD-plate with controls for amplification and detection.

Positive Control

The KRAS Mutant Control result must be 'Valid' for both working MMX1 and working MMX2. If the **KRAS MC** results are consistently invalid, contact your local Roche office for technical assistance.

Negative Control

The negative control (**NEG CT**) result must be 'Valid' for both working MMX1 and working MMX2. If the **NEG CT** results are consistently invalid, contact your local Roche office for technical assistance.

Calibrator

The KRAS Calibrator (**KRAS CAL**) result must be 'Valid' for both working MMX1 and working MMX2. If the **KRAS CAL** results are consistently invalid, contact your local Roche office for technical assistance.

PROCEDURAL PRECAUTIONS

As with any test procedure, good laboratory technique is essential to the proper performance of this assay. Due to the high analytical sensitivity of this test, care should be taken to keep reagents and amplification mixtures free of contamination.

PROCEDURAL LIMITATIONS

1. The **cobas** KRAS Test has only been validated for use with Colorectal Cancer FFPET Specimens.
2. The **cobas** KRAS Test has only been validated using the **cobas**® DNA Sample Preparation Kit (Roche M/N: 05985536190).
3. Detection of a mutation is dependent on the number of copies present in the specimen and may be affected by specimen integrity, amount of isolated DNA, and the presence of interfering substances.
4. Reliable results are dependent on adequate specimen fixation, transport, storage and processing. Follow the procedures in the **cobas**® DNA Sample Preparation Kit Instructions for Use (M/N 05985536190), this Instructions for Use, and in the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance.
5. The addition of AmpErase enzyme into the **cobas** KRAS Test Master Mix enables selective amplification of target DNA; however, good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary to avoid contamination of reagents.
6. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas**® 4800 System.
7. Only the **cobas**® z 480 analyzer has been validated for use with this product. No other thermal cycler with real-time optical detection can be used with this product.
8. The effects of other potential variables such as specimen fixation variables have not been evaluated.
9. Though rare, mutations within the regions of the genomic DNA of the KRAS gene covered by the **cobas** KRAS Test's primers and/or probes may result in failure to detect the presence of a mutation. Samples with results reported as "No Mutation Detected" may harbor KRAS mutations not detected by the assay.
10. The presence of PCR inhibitors may cause false negative or invalid results.
11. Though rare (< 0.2%[24]), the **cobas** KRAS Test shows results of "No Mutation Detected" for some complex and multiple mutations of codon 12/13 and codon 61, and demonstrates limited cross-reactivity for mutations flanking codon 12/13 on exon 2 and codon 61 on exon 3, including codon 59. In the case of a sample with a mutation in the flanking regions, the test may report a result of "Mutation Detected".
12. Patients may have additional KRAS mutations (A59X, K117X, A146X) which are not detected by the test. These mutations combined have a prevalence of 0.64% in CRC[24]. The **cobas** KRAS Test does not detect NRAS mutations.
13. The **cobas** KRAS Test was verified for use with 50 ng of DNA per reaction well. DNA input amounts lower than 50 ng per reaction well are not recommended.
14. The **cobas** KRAS Test is a qualitative test. The test is not for quantitative measurements of percent mutation level.
15. The procedure described above must be followed to detect ≥ 5% mutant sequences in a background of wild-type DNA for the KRAS mutations detected by the **cobas** KRAS Test. [24]
16. Pipetting from the bottom of the elution tube may disrupt the pellet and adversely affect test results. If a sample still has invalid sample results after following the procedure outlined in the "**Retesting of Specimens with Invalid Results**" section, do the following: after the elution step is complete, spin the eluted sample for 1 minute at 8,000 *x g* and transfer a portion of the supernatant into a new tube (1.5-mL RNase/DNase-free microcentrifuge tube) without disturbing the pellet at the bottom of the tube. Leave approximately 20 μL of supernatant at the bottom of the original tube in order to avoid the pellet, which may not be visible. Proceed to the "**AMPLIFICATION AND DETECTION**" section.

NON-CLINICAL PERFORMANCE EVALUATION

Analytical Sensitivity – Limit of Blank

To assess performance of the **cobas** KRAS Test in the absence of template and to ensure that a blank sample does not generate an analytical signal that might indicate a low concentration of mutation, no DNA template samples and DNA extracted from CRC FFPET KRAS wild type specimens were evaluated. Only "No Mutation Detected" results were observed in the no DNA template samples and in the presence of KRAS wild type DNA.

Analytical Sensitivity Using FFPET Specimen Blends

Multiple CRC FFPET specimen DNA extracts for specific codon 12 mutants (G12A, G12C, G12D, G12S, G12V, G12R), one codon 13 mutant (G13D) and one codon 61 mutant (Q61Hc) were blended with KRAS wild-type FFPET specimen extracts to achieve mutant sequences at approximately 10%, 5%, 2.5%, and 1.25% mutation level. The final mutation levels for all specimens were verified by a massively parallel sequencing (MPS) method that was validated for detecting the specific codon 12 mutants (G12A, G12C, G12D, G12S, G12V, G12R) and codon 13 mutant (G13D) and codon 61 (Q61Hc). Each specimen blend was diluted to 2 ng/µL at the time of testing (50.0ng/25 µL). Serial dilutions of each specimen blend were prepared and eight (8) replicates of each codon 12 and codon 13 mutant panel member were tested using each of 3 **cobas** KRAS Test kit lots (n=24 per panel member). Twenty replicates of each of the codon 61 mutant panel members were tested using 3 **cobas** KRAS Test kit lots (n=60 per panel member). The sensitivity of each sample was determined by the lowest amount of DNA that gave a KRAS "Mutation Detected" rate in at least 95% of the replicates, shown in Table 3. This study demonstrates that the **cobas** KRAS Test can detect KRAS codon 12, 13 and 61 mutations at approximately 5% mutant sequences using the standard input of 2 ng/µL.

Table 3

Sensitivity of the cobas KRAS Test using CRC Specimen FFPET Blends

KRAS Mutation	**Codon 12**						**Codon 13**	**Codon 61**
	G12A	**G12C**	**G12D**	**G12R**	**G12S**	**G12V**	**G13D**	**Q61Hc**
Targeted Level	2.5%	2.5%	1.25%	5.0%	2.5%	2.5%	1.25%	2.5%
LOD	2.93%	2.61%	1.64%	5.78%	2.55%	2.48%	1.67%	2.9%

Correlation to Reference Method

A study was conducted to compare the results of the **cobas** KRAS Test to Sanger sequencing (reference method) using 94 procured CRC FFPET specimens. A 5-µm section was processed to isolate DNA from each tumor specimen using one **cobas**® DNA Sample Preparation kit lot and the extracted DNA was tested with each of two reagent lots of the **cobas** KRAS Test. Tumor stage information, results of bi-directional Sanger sequencing and results of **cobas** KRAS Test for the 94 specimens are summarized in the tables below.

Table 4

Tumor Stage vs. Sanger Sequencing

Tumor Stage	**2X Bi-directional Sanger Sequencing Results**				**Total**	**% of Total**
	codon 12	**codon 13**	**codon 61**	**wild-type**		
Stage I	1	4	0	3	8	8.5%
Stage II	2	8	4	16	30	31.9%
Stage III	5	6	3	20	34	36.2%
Stage IV	7	5	1	9	22	23.4%
Total	15	23	8	48	94	100.0%

* 2x Bi-directional Sanger sequencing refers to two reads of bi-directional Sanger sequencing, i.e., a total of 4 reads including 2 forward and 2 reverse reads.

Table 5

Comparison of the cobas KRAS Test with Bi-Directional Sanger Sequencing for Detection of KRAS Mutations in Codon 12/13 and Codon 61

		2X Bi-directional Sanger Sequencing			
		Codon 12/13 Mutant	**Codon 61 Mutant**	**NMD**	**Total**
	Codon 12/13 Mutant	37	0	3	40
cobas KRAS Test	**Codon 61 Mutant**	0	8	1	9
	NMD	1	0	44	45
	Total	38	8	48	94

Positive Percent Agreement (PPA) = 97.8% (95% CI = 88.5 to 100.0%)
Negative Percent Agreement (NPA) = 91.7% (95% CI = 80.0 to 97.7%)
Overall Percent Agreement (OPA) = 94.7% (95% CI = 88.0 to 98.3%)
NMD: No Mutation Detected

Discordant results between the **cobas** KRAS Test vs. 2X bi-directional Sanger sequencing were resolved by 454 sequencing and are shown in Table 6.

Table 6

Comparison of the cobas KRAS Test with Bi-Directional Sanger Sequencing Resolved by 454 Sequencing for Detection of KRAS Mutations in Codon 12/13 and Codon 61

			2X Bi-directional Sanger Sequencing+454		
		Codon 12/13 Mutant	**Codon 61 Mutant**	**NMD**	**Total**
	Codon 12/13 Mutant	40	0	0	40
cobas KRAS Test	**Codon 61 Mutant**	0	8	1*	9
	NMD	0	0	45	45
	Total	40	8	46	94

Positive Percent Agreement (PPA) = 100.0% (95% CI = 92.6 to 100.0%)
Negative Percent Agreement (NPA) = 97.8% (95% CI = 88.5 to 100.0%)
Overall Percent Agreement (OPA) = 98.9% (95% CI = 94.2 to 100.0%)
*Confirmed to be a codon 59 mutation
NMD: No Mutation Detected

Another study tested 188 colorectal cancer FFPET specimens with both the **cobas** KRAS Test and Sanger sequencing using two lots of the **cobas** KRAS Test kits stored at 2-8°C. Comparable results were obtained with this procured sample set, with a PPA of 96.6% (CI: 90.4-99.3%), an NPA of 93.0% (CI: 86.1-97.1%), and an OPA of 94.7% (CI: 90.4-97.4%).

Cross-Reactivity to other KRAS Mutations on Codon 13

The **cobas** KRAS Test has been shown to cross-react with the following mutations shown in Table 7. Plasmid constructs (n=4) and CRC FFPET (n=1) containing the rare mutations for codon 13 were blended with wild type genomic DNA to create approximately 5% mutant samples. Results demonstrated that the **cobas** KRAS Test cross-reacts to the following mutations at a 100% hit rate. Analytical performance of the **cobas** KRAS Test in detecting these mutations has not been evaluated.

Table 7

Mutations Determined to Cross-React with the cobas KRAS Test

Mutation	AA Change	COSMIC ID
c.37G>T	**G13C**	527
c.37G>A	**G13S**	528
c.37G>C	G13R	529
c.38G>C	**G13A**	533
c.38G>T	**G13V**	534

Bold = Tested with plasmids

Specificity – KRAS silent mutation, KRAS Homologs and Microorganisms

Specificity of the **cobas** KRAS Test was evaluated by the following studies:

- Testing KRAS silent mutation plasmids,
- Testing KRAS homolog plasmids,
- Testing colon-related microorganisms.

Cross-reactivity was also evaluated by determining whether or not the presence of KRAS silent mutation plasmids or KRAS homolog plasmids or colon-related microorganisms interfered with detection of KRAS codon 12, 13 and 61 mutations.

KRAS Silent Mutation Plasmids

Plasmid samples in a background of wild-type cell line DNA were prepared and tested for the following three KRAS codon 12 silent mutations: GGA, GGC, and GGG; three KRAS codon 13 silent mutations: GGA, GGT, and GGG. No cross-reactivity was observed with plasmids for KRAS codon 12 silent mutations or codon 13 silent mutations.

Plasmid blends of KRAS codon 12 or codon 13 at 5% mutation in a background of wild-type cell line DNA were prepared and tested in the presence of their respective silent mutation plasmids and no interference from the silent mutation plasmids was detected.

KRAS Homolog Plasmids

Samples containing each of the six KRAS Homolog plasmids (KRAS codon 12/13 pseudogene, codon 61 pseudogene, NRAS exon 2, NRAS exon 3, HRAS exon 2 and HRAS exon 3) in a background of wild-type cell line DNA were prepared and tested in triplicate using the **cobas** KRAS Test. No cross reactivity was observed with any of the plasmid samples.

Plasmid blends of KRAS codon 12, codon 13 and codon 61 at 5% mutation in a background of wild-type cell line DNA were prepared and tested in the presence of their respective homolog plasmids and no interference from the homolog plasmids was detected.

Colon-related Microorganisms

The following colon-related microorganisms were found not to cross react in the **cobas** KRAS Test when added to six KRAS codon 12, one codon 13, two codon 61 and one wild-type specimens at 1 x 10^6 colony forming units during the tissue lysis step:

1. *Bacteroides caccae*
2. *Prevotella intermedia*
3. *Escherichia coli (E. coli)*

The tested microorganisms also did not interfere with the detection of KRAS codon 12, codon 13 or codon 61 mutations when 1 x 10^6 colony forming units were added during the tissue lysis step of a specimen containing a KRAS mutation at levels ranging from 14-40%.

Interference

Triglycerides ($\leq$ 37mM, CLSI recommended high concentration[25]) and hemoglobin ($\leq$ 2 mg/mL, CLSI recommended high concentration[25]) have been shown not to interfere with the **cobas** KRAS Test when the potential interfering substance was added to the lysis step during the specimen preparation procedure.

Necrotic Tissue

CRC FFPET specimens with necrotic tissue content up to 50% for KRAS mutant and 70% in KRAS wild-type specimens have been shown not to interfere with the call results for the **cobas** KRAS Test.

Repeatability

Repeatability of the **cobas** KRAS Test was assessed using eight colorectal cancer FFPET specimens, including 2 codon 12, 2 codon 13, 2 codon 61 KRAS mutant specimens, and 2 KRAS wild type specimens. The specimens were tested in duplicate by two operators, using two different reagent lots and four **cobas**® z 480 analyzers over 4 days (n=32/specimen) at one site. The **cobas** KRAS Test had a correct call accuracy of 100% (256/256) for all days, specimens, replicates, operators and reagent lots combined.

Specimen Handling Reproducibility

The reproducibility of the DNA Sample Preparation Kit was examined using sections taken from three CRC FFPET specimen blocks, one containing a codon 12 mutation (G12D, GGT>GAT), one containing a codon 13 mutation (G13D, GGC>GAC), and one that is wild-type for KRAS mutation. From each of the three specimens, thirty-six (36) 5-µm sections were obtained. Each of three external sites tested twelve (12) sections for each specimen over six (6) non-consecutive days. On each test day, one operator from each site isolated, quantified and tested the DNA from two FFPET curl sections for each specimen using one lot of **cobas**® DNA Sample Preparation Kit and one lot of the **cobas** KRAS Test kit. All testing was performed on the **cobas**® z 480 analyzer with the KRAS Analysis Package. One lot of the **cobas** KRAS Test kit reagents was used in this study, in combination with three lots of the DNA Sample Preparation kit at each site. All runs performed at the 3 sites were valid. All mutant and wild-type specimen results were valid and yielded the expected call result (correct call =100%, 36/36 for each specimen), supporting the reproducibility for the **cobas** KRAS Test at the pre-analytical step of DNA isolation.

CLINICAL PERFORMANCE EVALUATION

Clinical Reproducibility

An external study was performed to assess the reproducibility of the **cobas** KRAS Test with a 17 member panel of DNA samples extracted from CRC FFPET sections of KRAS wild type (WT) and mutant type (MT) tumor specimens, tested in duplicate, across 3 testing sites with 2 operators per site, 3 reagent lots and 5 non-consecutive testing days. The panel included six codon 12 mutations, one codon 13 mutation, and one codon 61 mutation along with a WT DNA sample. Each mutation was represented in duplicate at the limit of detection (LOD) and 3x LOD. Two replicates of the panel at desired concentrations were used in each run. Panels were provided to each of the 3 testing sites in a blinded fashion. Operators performed amplification and detection with the **cobas**® z 480 instrument using the **cobas** KRAS Test kits. One panel tested by a given operator at a given site was considered one run. Each run included 2 replicates of each of the 17 specimens in the panel. Each operator completed 5 valid runs with each of 3 reagent lots, performed runs on non-consecutive days, and performed runs separately from the other operator at the site. Testing was completed with one lot before testing began with another lot.

Overall 97.8% of runs (90/92) were valid. A total of 3,060 tests were performed in 90 valid runs. From the valid runs, 99.6% of test results (3,048/3,060) were valid. The agreement in the wild type panel was 100% with ‘No Mutation Detected’ results in all 175 valid tests. For the Codon 12, 13, and 61 panel members at both 1 x LOD and 3 x LOD, agreement was 100%. Across all variance components (i.e., lot, site/instrument, operator, day, and within run), the overall coefficient of variation ranged from 5.1% to 13.3% across all panel members.

Table 8

Overall Estimates of Agreement by Panel Member

Panel Member	Number of Valid Tests	Agreement N	Agreement % (95% CI)*
Wild Type	175	175	100.0 (97.9, 100.0)
Codon 12 - Mutation 12D – LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12V – LOD	178	178	100.0 (97.9, 100.0)
Codon 12 - Mutation 12C – LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12A – LOD	179	179	100.0 (98.0, 100.0)
Codon 12 - Mutation 12S – LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12R – LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12D - 3 x LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12V - 3 x LOD	179	179	100.0 (98.0, 100.0)
Codon 12 - Mutation 12C - 3 x LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12A - 3 x LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12S - 3 x LOD	180	180	100.0 (98.0, 100.0)
Codon 12 - Mutation 12R - 3 x LOD	180	180	100.0 (98.0, 100.0)
Codon 13 - Mutation 13D – LOD	180	180	100.0 (98.0, 100.0)
Codon 13 - Mutation 13D - 3 x LOD	180	180	100.0 (98.0, 100.0)
Codon 61 – Mutation 61H – LOD	180	180	100.0 (98.0, 100.0)
Codon 61 – Mutation 61H – 3 x LOD	177	177	100.0 (97.9, 100.0)

Note: Results are included as agreement when a valid test of Mutant Type panel member has a result of 'Mutation Detected or when a valid test of Wild Type panel member has a result of 'No Mutation Detected'.

[*] 95% CI = 95% exact binomial confidence interval.

KRAS Mutation Results by Different Test Methods

The performance of the **cobas** KRAS Test compared with Sanger sequencing and an FDA-approved IVD test was evaluated for detection of mutations in codons 12, 13 and 61 of the KRAS gene in CRC tumor specimens from subjects enrolled in Roche study NO16968 (the Roche study). Formalin-fixed paraffin-embedded tissue (FFPET) samples from 398 patients from this trial and 82 CRC supplemental samples were tested.

A total of 480 tumor samples were available for the study. Nineteen samples from the Roche study cohort were not eligible for KRAS mutation testing. Of them, 15 were not eligible due to no tumor content and 4 could not be confirmed as CRC by pathology evaluation. A total of 461 samples were eligible and tested for KRAS mutations by the 3 methods.

All of the 461 eligible samples were tested by the **cobas** KRAS Test, Sanger sequencing and an FDA-approved IVD test (Table 9). If the initial test result was invalid by any of the three tests, re-testing by the **cobas** KRAS Test and the other FDA-approved IVD test was performed up to two times. However, for Sanger sequencing, the number of attempts were not limited unless there was insufficient remaining sample quantity for re-testing. The **cobas** KRAS Test had an invalid rate of 5.4%. Sanger sequencing had an invalid rate of 4.6% and the FDA-approved IVD test had an invalid rate of 10.8%, 8% of which did not qualify in the DNA sample assessment step and 2.8% gave invalid results for mutation testing. Among samples with valid results, the **cobas** KRAS Test and the FDA-approved IVD test had similar KRAS mutation-positive rates for mutations in codon 12/13 (37.4% vs 36.3%, respectively), while Sanger sequencing had a lower positive rate for codon 12/13 (29.1%). The other FDA-approved IVD test does not have the ability of detecting mutations in codon 61, but the **cobas** KRAS Test and Sanger sequencing had similar KRAS mutation-positive rates for mutations in codon 61 (2.1% vs 2.5%, respectively).

Table 9

KRAS Mutation Result by Different Testing Methods

	cobas KRAS Test	FDA approved IVD test	Sanger Sequencing
Number of Samples Tested	461	461	461
Invalid result	25 (5.4%)	50 (10.8%)	21 (4.6%)
Valid result	436 (94.6%)	411 (89.2%)	440 (95.4%)
No Mutation Detected	264 (60.6%)	262 (63.7%)	301 (68.4%)
Mutation Detected	172 (39.4%)	149 (36.3%)	139 (31.6%)
Codon 12/13*	163 (37.4%)	149 (36.3%)	128 (29.1%)
Codon 61	9 (2.1%)	N.A.**	11 (2.5%)

Note: 37 samples with tumor content ≥20% failed sample assessment for the FDA-approved IVD test. These samples did not go through the next step of mutation detection and were counted as invalid results.

Note: Validity of the test results were based on the validity of both codon 12/13 and codon 61 for **cobas** KRAS Test and Sanger Sequencing and only on codon 12/13 for the FDA approved test.

* Mutation detected for codon 12/13 may include samples with mutations in both codon 12/13 and codon 61.

**The FDA approved test does not have the ability to detect codon 61 mutation.

Agreement between the cobas KRAS Test with Sanger Sequencing and Other FDA Approved Test in Detection of KRAS Mutation in Codon 12/13

The agreement of the **cobas** KRAS Test using Sanger sequencing or the FDA-approved IVD test as the reference method for detection of mutations in codon 12/13 is presented in Table 10. The PPA between the **cobas** KRAS Test and Sanger sequencing was 96.9% (95% CI: 92.2% to 98.8%), and the NPA was 88.7% (95% CI: 84.7% to 91.8 %). The PPA between the **cobas** KRAS Test and the FDA-approved IVD test was 93.3% (95% CI: 88.1% to 96.3%), and the NPA was 96.5% (95% CI: 93.5% to 98.1%).

Table 10

Comparison of the cobas KRAS Test with Reference Methods for Detection of KRAS Mutation in Codon 12/13

cobas KRAS Test	Reference Method							
	Sanger Sequencing				FDA-approved IVD test			
	Mutation Detected for Codon 12/13	No Mutation Detected for Codon 12/13	Invalid	Total	Mutation Detected for Codon 12/13	No Mutation Detected for Codon 12/13	Invalid	Total
Mutation Detected for Codon 12/13	124	34	5	163	139	9	15	163
No Mutation Detected for Codon 12/13	4	268	2	274	10	248	16	274
Invalid	0	19	5	24	0	5	19	24
Total	128	321	12	461	149	262	50	461
PPA (95% CI)	96.9% (92.2%, 98.8%)				93.3% (88.1%, 96.3%)			
NPA (95% CI)	88.7% (84.7%, 91.8%)				96.5% (93.5%, 98.1%)			
OPA (95% CI)	91.2% (88.1%, 93.5%)				95.3% (92.8%, 97.0%)			

Note: Validity of the test results were based on the validity of codon 12/13 ONLY for all 3 methods.

* Mutation detected for codon 12/13 may include samples with mutations in both codon 12/13 and codon 61.

** No Mutation Detected for codon 12/13 may include samples with mutation only in codon 61.

Predictive Values of the cobas KRAS Test for Detection of KRAS Mutations in Codon 12/13

The predictive values of the **cobas** KRAS Test for detection of codon 12/13 were calculated by combining the PPA and NPA of **cobas** KRAS Test relative to a comparator method together with the prevalence of a KRAS “Mutation Detected for codon 12/13” result by the comparator method in published clinical studies for cetuximab or panitumumab.[26-29] The positive and negative predictive values (PPV and NPV) of **cobas** KRAS Test refer to the predictive values of “Mutation Detected for codon 12/13” and “No Mutation Detected for codon 12/13” results for the comparator method, respectively.[30] The clinical performance was summarized by the quantity of PPV+NPV−1; this quantity has been called the attenuation factor.[30]

Table 11

Attenuation Factors for cobas KRAS Test (codon 12/13)

Data Source[Ref]	Comparator Method	PPV (95% CI)	NPV (95% CI)	Attenuation Factor (95% CI)
Cetuximab[26]	Sanger Sequencing	0.858 (0.811, 0.902)	0.975 (0.946, 0.994)	83.3% (77.7, 88.3)
Cetuximab[31]	FDA-approved IVD test	0.957 (0.927, 0.981)	0.945 (0.909, 0.978)	90.2% (85.6, 94.4)
Panitumumab[29,32]	FDA-approved IVD test	0.949 (0.914, 0.977)	0.956 (0.927, 0.981)	90.4.% (86.1, 94.4)

Agreement between the cobas KRAS Test and Sanger Sequencing for Codon 61

The agreement of the **cobas** KRAS Test and Sanger sequencing for the detection of mutations at codon 61 is presented in Table 12. With only valid results, PPA is estimated as 100% (95% CI: 70.1%, 100%) and NPA is estimated as 99.3% (95% CI: 97.9%, 99.8%).

Table 12

Agreement between cobas KRAS Test and Sanger Sequencing (codon 61)

		Sanger Sequencing			**Total**
		Mutation Detected for Codon 61	**No Mutation Detected for Codon 61**	**Invalid**	
cobas KRAS Test	**Mutation Detected for Codon 61**	9	3	0	12
	No Mutation Detected for Codon 61	0	420	4	424
	Invalid	2	13	10	25
	Total	11	436	14	461
With only Valid Result	**PPA (95% CI)**	100.0% (70.1%, 100.0%)			
	NPA (95% CI)	99.3% (97.9%, 99.8%)			

Note: Validity of the test results were based on the validity of both codon 12/13 and codon 61.

LIST OF RESULT FLAGS

Result flags may be found under the **Results** tab. The source of a flag is indicated in the flag code as outlined in Table 13.

Table 14 lists all result flags of the system that are user relevant.

Table 13 Flag Source

Flag Code Starts With	Flag Source	Example
M	Multiple or other reason	M6
R	Result Interpretation	R20
Z	Analyzer	Z1

Table 14 List of System Flags

Flag Code	Severity	Description	Recommended Action
M1	Error	Error: Software error occurred. For more information, refer to alarm messages and log files.	Refer to alarm messages and log files. If this does not help, contact Roche Service.
M2	Information	Information: Run was aborted by the user.	None. Flag is for information only.
M6	Information	Information: Communication with **cobas**® z 480 was lost. Run was recovered after the communication was re-established.	None. Flag is for information only. For details, see run recovery in the **cobas**® z 480 analyzer Instrument Manual.
R251-R252 R261-R262	Error	Mutant control out of range.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use. These flag codes indicate that one or more melting temperatures of the mutant control were outside of the established range. This may occur in the event of: • Incorrect preparation of working master mix. • Pipetting error when adding the working master mix into one or both mutant control wells of the microwell plate (positions A01 for R251-252 and A02 for R261-262). • Pipetting error when adding the mutant control in one or both mutant control wells of the microwell plate (positions A01 for R251-252 and A02 for R261-262). • No addition of the mutant control in one or both mutant control wells of the microwell plate (positions A01 for R251-252 and A02 for R261-262). • DNA contamination.

Flag Code	Severity	Description	Recommended Action
R253-R255 R263-R265	Error	Mutant control out of range.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use. These flag codes indicate that the melting observed peak height values and other parameters for the mutant control were outside of the established range. This may occur in the event of: • Incorrect preparation of working master mix. • Pipetting error when adding the working master mix into one or both mutant control wells of the microwell plate (positions A01 for R253-255 and A02 for R263-265). • Pipetting error when adding the mutant control in one or both mutant control wells of the microwell plate (positions A01 for R253-255 and A02 for R263-265). • No addition of the mutant control in one or both mutant control wells of the microwell plate (positions A01 for R253-255 and A02 for R263-265).
R256-R257 R266-R267	Error	Negative control out of range.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use. These flag codes indicate that one or more peak height values are above the established threshold for the negative control. This may occur in the event of DNA contamination.
R258, R268	Error	Calibrator out of range.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use. These flag codes indicate that the melting temperature of the calibrator was outside of the established range. This may occur in the event of: • Incorrect preparation of working master mix. • Pipetting error when adding the working master mix into one or both calibrator wells of the microwell plate (position C01 for R258 and C02 for R268). • Pipetting error when adding the calibrator in one or both calibrator wells of the microwell plate (position C01 for R258 and C02 for R268). • No addition of the calibrator in one or both calibrator wells of the microwell plate (position C01 for R258 and C02 for R268). • DNA contamination.

Flag Code	Severity	Description	Recommended Action
R259, R269	Error	Calibrator out of range.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use. These flag codes indicate that the observed wild-type peak height values for the calibrator were below the established threshold. This may occur in the event of: • Incorrect preparation of working master mix. • Pipetting error when adding the working master mix into one or both calibrator wells of the microwell plate (position C01 for R259 and C02 for R269). • Pipetting error when adding the calibrator in one or both calibrator wells of the microwell plate (position C01 for R259 and C02 for R269). • No addition of the calibrator in one or both calibrator wells of the microwell plate (position C01 for R259 and C02 for R269).
R260, R270	Error	Calibrator out of range.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use. These flag codes indicate that observed peak height value for the mutant peak was above the pre-established limit. This may occur in the event of DNA contamination.
R271-R278	Error	Mutant control not detected.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use. These flag codes indicate that the melting temperatures were invalid or negative for the mutant control (for the mutant and/or wild-type peak). This may occur in the event of: • Incorrect preparation of working master mix. • Pipetting error when adding the working master mix into one or both mutant control wells of the microwell plate (positions A01 for R271-274 and A02 for R275-278). • Pipetting error when adding the mutant control in one or both mutant control wells of the microwell plate (positions A01 for R271-274 and A02 for R275-278). • No addition of the mutant control in one or both mutant control wells of the microwell plate (positions A01 for R271-274 and A02 for R275-278).

Flag Code	Severity	Description	Recommended Action
R279-R282 R299-R302	Error	No result detected.	Repeat the sample. Refer to the *Interpretation of Results* section of the test-specific instructions for use. These flag codes indicate that both mutant and wild-type melting temperatures were undetected. This may occur in the event of: • Incorrect preparation of working master mix. • Pipetting error when adding the working master mix into one or more reaction wells of the microwell plate. • Pipetting error when adding the DNA template in one or more wells of the microwell plate. • Insufficient amplifiable DNA available.
R283-R297 R303-R317	Error	Result out of range.	Repeat the sample. Refer to the *Interpretation of Results* section of the test-specific instructions for use. These flag codes indicate that either mutant or wild-type melting temperatures and/or peak height values were out of the established range. This may occur in the event of: • Incorrect preparation of working master mix. • Pipetting error when adding the working master mix into one or more reaction wells of the microwell plate. • Pipetting error when adding the DNA template in one or more wells of the microwell plate. • Insufficient amplifiable DNA available.
Z1	Error	Error: Error occurred on cobas z 480. Run was aborted.	Contact Roche Service.

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Doc Rev. 6.0 05/2024	Update hazard information. Updated the harmonized symbol page. Added **Technical support** section. Updated **Trademarks and patents** section, including the link. Added IVD symbol. Updated **cobas**® branding. Inserted Rx only text above legal manufacturer. Updated to current economic operators. Removed “Distributed by” symbol. Please contact your local Roche Representative if you have any questions.

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Age/DOB	Age or Date of Birth
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Assigned Range [copies/mL]	Assigned Range (copies/mL)
Assigned Range [IU/mL]	Assigned Range (IU/mL)
EC REP	Authorized representative in the European Community
BARCODE	Barcode Data Sheet
LOT	Batch code
☣	Biological risks
REF	Catalogue number
CE	CE marking of conformity; this device is in conformity with the applicable requirements for CE marking of an in vitro diagnostic medical device
Collect Date	Collect date
i	Consult instructions for use
Σ	Contains sufficient for <*n*> tests
CONTENT	Content of kit
CONTROL	Control
	Date of manufacture
	Device for near-patient testing
	Device for self-testing
	Device not for near-patient testing
	Device not for self-testing
	Distributor *(Note: The applicable country/region may be designated beneath the symbol)*
	Do not re-use
♀	Female
?	For IVD performance evaluation only
GTIN	Global Trade Item Number
	Importer
IVD	In vitro diagnostic medical device
LLR	Lower Limit of Assigned Range
♂	Male
	Manufacturer
CONTROL −	Negative control
NON STERILE	Non-sterile
?	Patient Name
#	Patient number
	Peel here
CONTROL +	Positive control
QS copies / PCR	QS copies per PCR reaction, use the QS copies per PCR reaction in calculation of the results.
QS IU/PCR	QS IU per PCR reaction, use the QS International Units (IU) per PCR reaction in calculation of the results.
SN	Serial number
Site	Site
Procedure Standard	Standard Procedure
STERILE EO	Sterilized using ethylene oxide
	Store in dark
	Temperature limit
TDF	Test Definition File
↑↑	This way up
Procedure UltraSensitive	Ultrasensitive Procedure
UDI	Unique Device Identifier
ULR	Upper Limit of Assigned Range
Urine Fill Line	Urine Fill Line
Rx only	For USA: Caution: Federal law restricts this device to sale by or on the order of a physician.
⧗	Use-by date