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 intended for the identification of mutations in codons 12, 13 and 61 of the KRAS gene in DNA derived from formalin-fixed paraffin-embedded human colorectal (CRC) and non-small cell lung cancer (NSCLC) tissues.

SUMMARY AND EXPLANATION OF THE TEST

KRAS Protein:

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, 13 or 61, which occur in a variety of human malignancies, including colorectal cancer (CRC) and non-small cell lung cancer (NSCLC), lock the enzyme in the GTP-bound, activated form, resulting in constitutive signaling and thereby contributing to the oncogenic process.[1]

CRC:

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); KRAS mutations in other codons (e.g., codon 61) are less common (1%-4% of mutations). Although codon 61 mutations are infrequent, they have been shown to result in constitutive activation of KRAS just as codon 12 and 13 mutations do,[4] and published data indicate that codon 61 mutations predict non-response to anti-EGFR monoclonal antibody therapy.[5-6]

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.[7]

However, there is 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.[8-9] The supporting evidence for these findings comes from:

- Retrospective analyses of single-arm studies[10-11]
- Retrospective analyses of randomized studies[12-13]
- Prospective randomized studies[14]

As a consequence of these studies, KRAS 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]

NSCLC:

A number of recurring molecular abnormalities have been identified in NSCLC. Point mutations of the KRAS gene have been found in 10% to 30% of advanced NSCLC, primarily but not exclusively in adenocarcinomas.[20] Most frequently, somatic KRAS/RAS mutations affect codons 12, 13, and 61, resulting in accumulation of active KRAS/RAS protein in the cell and subsequent activation of signaling pathways involved in malignant transformation.

Published literature suggests that patients with KRAS positive advanced NSCLC have a worse prognosis[21] and may not benefit from adjuvant chemotherapy[22] or EGFR TKI therapy.[23] It has also been found that patients whose tumors harbor EGFR mutations do not harbor KRAS mutations and vice versa[24] Thus such patients represent a subset of NSCLC patients with high unmet clinical need due to fewer treatment options available compared with patients whose tumors are KRAS wild type.

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, and thus identify patients with advanced CRC who are unlikely to benefit from therapy with anti-EGFR monoclonal antibodies or patients with NSCLC who may not benefit from adjuvant chemotherapy or erlotinib.

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 two oligonucleotide probes 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 divalent metal ion and excess dNTP, 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. Amplification occurs only in the regions of the KRAS gene between the primer pairs; the entire KRAS gene is not amplified.

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. Amplicon with a perfect match to the probe (wild-type) melt at a higher temperature than amplicon 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).[25] 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 amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon 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 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy 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. P261: Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray. P272: Contaminated work clothing should not be allowed out of the workplace. 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.
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 <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. P261: Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray. P272: Contaminated work clothing should not be allowed out of the workplace. 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.
KRAS OM2 **(KRAS Oligo Mix 2)**	Tris-HCl buffer EDTA Poly-rA RNA (synthetic) 0.1% ProClin® 300 preservative <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. P261: Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray. P272: Contaminated work clothing should not be allowed out of the workplace. 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.

cobas® KRAS Mutation Test (KRAS) **24 Tests** (M/N: 05852170190)			
Kit components	**Reagent ingredients**	**Quantity per kit**	**Safety symbol and warning[a]**
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.

WARNINGS AND PRECAUTIONS

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

B. This test is for use with formalin-fixed paraffin-embedded colorectal and non-small cell lung 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[26] and in the CLSI Document M29-A3.[27]

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 z** 480 analyzer instrument manual.

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 pipettor 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 PROVIDED

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

KRAS MIX
(Reaction Mix) (Cap with Natural Button)

MGAC
(Magnesium acetate) (Cap with Yellow Button)

KRAS OM1
(KRAS Oligo Mix 1) (Cap with White Button)

KRAS OM2
(KRAS Oligo Mix 2) (Cap with Gold Button)

KRAS MC
(KRAS Mutant Control) (Cap with Red Button)

KRAS CAL
(KRAS Calibrator) (Cap with Purple Button)

DNA SD
(DNA Specimen Diluent)

MATERIALS REQUIRED BUT NOT PROVIDED

- **cobas®** DNA Sample Preparation Kit (Roche M/N 05985536190)
- **cobas®** 4800 System microwell plate (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
- Pipette aid (Drummond M/N: 4-000-100 or equivalent)
- 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
- Freezer capable of -25°C to-15°C storage

* *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 2.0 or higher
- KRAS Analysis 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 and non-small cell lung cancer 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.[28]

C. Specimen Storage

FFPET specimens may be stored at 15ºC to 30°C for up to 12 months after the date of tissue collection. 5-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 are to be used in the cobas KRAS Test. Any specimen containing less than 10% tumor content 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) per 96 well AD-plate. 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 sample using two 5-µm FFPET sections. For mounted samples, after deparaffinization, combine the tissue from both sections into one tube, immerse the tissue in TLB + PK from the cobas® DNA Sample Preparation Kit, and perform DNA isolation and quantitation. For unmounted samples, combine the tissue from both sections into one tube and perform deparaffinization, DNA isolation and quantitation. If the DNA stock is still < 4 ng/µL, request another FFPET sample section from the referring clinical site.

NOTE: 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.

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:

Dilution Calculation for DNA Stock Concentrations from 4 ng/µL to 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 MMX 1 and for MMX 2) 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 MMX 1 and for MMX 2) 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 MMX 1 and KRAS MMX 2 may appear clear to yellow. This does not affect the performance of the reagent.

Prepare two bulk working MMX, one containing **KRAS MMX 1** and the other containing **KRAS MMX 2** 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 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 tune, 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 MMX 1 (containing **KRAS OM1**) to the AD-plate wells in the odd-numbered columns (1, 3, 5, etc.)
- Add working MMX 2 (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 – Operator's Manual or **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	Codon 12/13 or Codon 61 (both may be present)	Mutation detected in KRAS codon 12/13 or 61, or both.
Mutation Not Detected or 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 Mutation Not Detected or No Mutation Detected result does not preclude the presence of a mutation in the KRAS 12/13 or 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 (MMX 1 and MMX 2)**" 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** set of KRAS Test Mutant Control (**KRAS MC**), negative control (**NEG CT**) and KRAS Calibrator (**KRAS CAL**) for working MMX 1 and working MMX 2 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 MMX 1 or working MMX 2 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 MMX 1 and working MMX 2. 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 MMX 1 and working MMX 2. 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 MMX 1 and working MMX 2. 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. Test only the indicated specimen types. The **cobas** KRAS Test has only been validated for use with Colorectal cancer (CRC) and Non-Small Cell Lung Cancer (NSCLC) 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), in 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. Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to the next, users perform method correlation studies in their laboratory to qualify technology differences.
9. The effects of other potential variables such as specimen fixation variables have not been evaluated.
10. 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.
11. The presence of PCR inhibitors may cause false negative or invalid results.

12. Though rare (< 0.2%[29]), the **cobas** KRAS Test shows results of "Mutation Not 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".

13. 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[29]. The **cobas** KRAS Test does not detect NRAS mutations.

14. 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.

15. The **cobas** KRAS Test is a qualitative test. The test is not for quantitative measurements of percent mutation level.

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 to avoid the pellet, which may not be visible. Proceed to the "**AMPLIFICATION AND DETECTION**" section.

17. The procedure described above must be followed to detect ≥ 5% mutant sequences in a background of wild-type DNA for the KRAS mutations [29] in Table 3.

Table 3
Mutations Detected by the cobas KRAS Test

Mutation	AA Change	COSMIC ID
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
c.37G>T	13C	527
c.37G>A	**13S**	528
c.37G>C	13R*	529
c.38G>A	13D	532
c.38G>C	**13A**	533
c.38G>T	**13V**	534
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	61H (CAC)	554
c.183A>T	61H (CAT)	555

*Not tested for NSCLC FFPET specimens
Bold = Tested for plasmids

NON-CLINICAL PERFORMANCE EVALUATION

I. COLORECTAL CANCER (CRC) TISSUE

Analytical Sensitivity

The analytical sensitivity of the **cobas** KRAS Test was performed using dilution panels prepared from four types of specimens:

- Cell line blend prepared by mixing DNA stocks obtained from a KRAS mutant cell line and a KRAS wild-type cell line.
- Plasmid blend prepared by mixing a plasmid containing the KRAS mutation and DNA stocks obtained from KRAS wild-type cell line.

- Specimen blends prepared by mixing DNA stocks obtained from KRAS mutant FFPET specimens and KRAS wild-type FFPET specimens.
- DNA stock extracted from an individual FFPET specimen.

All specimens used in this study were sequenced by 454 Genome Sequencer FLX Titanium (454 sequencing) in order to determine the percent mutation of each specimen.

Analytical Sensitivity of the cobas KRAS Test Using Cell Line or Plasmid Blends

DNA from colorectal cell lines containing either the KRAS exon 2 codon 12 or 13 mutation were extracted and blended with DNA extracts from a KRAS wild-type cell line to achieve a sample at ~5% mutation, verified by 454 sequencing. Three separate dilution panels contained the following dilutions (50.0, 25.0, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 ng/25 µL). Twenty-four (24) replicates of each panel member were tested, using each of 3 **cobas** KRAS Test kit lots (72 replicates total). Sensitivity was determined by the lowest amount of DNA that gave a KRAS "Mutation Detected" rate of at least 95%, shown in Table 4.

Table 4
Sensitivity of the cobas KRAS Test using CRC Cell Line or Plasmid Blend

KRAS Mutation	Specimen Type	Percent Mutation*	Amount of DNA in the Panel Member (ng/25 µL) to achieve ≥ 95% "Mutation Detected" Rate (N=72 replicates)
Codon 12 (Exon 2)	Cell Line Blend	5.3%	0.8
Codon 13 (Exon 2)	Cell Line Blend	4.9%	1.6
Codon 61 (Exon 3)	Plasmid Blend	5.8%	6.3

* Mean percent mutation by 454 sequencing

The Test gave a 95% "Mutation Detected" rate at 0.8 ng/25 µL, 1.6 ng/25 µL, and 6.3 ng/25 µL (1:64, 1:32, and 1:8 dilutions of the recommended DNA input of 50 ng/25 µL) for KRAS codon 12, 13, and 61 mutations, respectively. This would indicate that the test will detect the tested KRAS mutations when ~87% of the DNA is degraded or non-amplifiable due to the fixation process, assuming that the cell line and plasmid DNA blends contained 100% intact and amplifiable DNA.

Analytical Sensitivity Using FFPET Specimen and Specimen blends

KRAS codon 12, 13 and 61 mutant FFPET specimen DNA extracts were blended with KRAS wild-type FFPET specimen extracts to achieve samples at ~5% mutation level. One naturally occurring specimen was used to test the KRAS codon 12 mutation. The final mutation levels for all specimens were verified by 454 sequencing. Each of the specimen/specimen blends was diluted to produce the panel members (50.0, 25.0, 12.5, 6.3, 3.1, 1.6, 0.8, and 0.4 ng/25 µL). The 50 ng/25 µL panel member was not tested for the blend #2 for Exon 3, codon 61.

Eight (8) replicates of each panel member were run using each of 3 **cobas** KRAS Test kit lots (n=24/panel member). The sensitivity of each sample was determined by the lowest amount of DNA that gave a KRAS "Mutation Detected" rate of at least 95%, shown in Table 5.

Table 5
Sensitivity of the cobas KRAS Test using CRC Specimen and Specimen Blends

KRAS Mutation	**Specimen Type**	**Percent Mutation**	**Amount of DNA in the Panel Member (ng/25 µL) to achieve ≥ 95% "Mutation Detected" Rate (N=24 replicates)**
Codon 12 (Exon 2)	FFPET Specimen	4.3%	3.1
	FFPET Blend	4.2%	3.1
	FFPET Blend	4.7%	3.1
Codon 13 (Exon 2)	FFPET Blend	5.0%	3.1
	FFPET Blend	4.6%	1.6
	FFPET Blend	7.2%	1.6
Codon 61 (Exon 3)	FFPET Blend	4.4%	3.1
	FFPET Blend	5.5%	3.1
	FFPET Blend	3.8%	6.3

This study demonstrates that the **cobas** KRAS Test can detect KRAS codon 12, 13 and 61 mutations at the ~5% mutation level using the standard input of 50 ng/25 µL. The ability of the test to detect the mutation at lower DNA input levels demonstrates that the specimens can contain degraded or non-amplifiable DNA from the fixation process and still be detected.

Correlation to Reference Method

One hundred and eighty-eight (188) colorectal cancer FFPET specimens were tested using each of 2 lots of **cobas** KRAS Test kits. Comparison testing by 2X Bi-directional Sanger sequencing was performed on all specimens. Discordant results between the **cobas** KRAS Test and 2X Bi-directional Sanger sequencing were resolved using 454 sequencing.

cobas KRAS Test and 2X Bi-directional Sanger Sequencing Results

Specimen information and results of 2X bi-directional Sanger sequencing for the 188 specimens are summarized in Table 6. Eighty-one (81) of the 188 specimens were KRAS codon 12/13 mutant and 7 were KRAS codon 61 mutant, while 107 of the specimens were either KRAS wild-type or KRAS non-codon 12/13 mutant by Sanger sequencing and 181 specimens were either KRAS wild-type or KRAS non-codon 61 mutant.

Table 6
Tumor Stage vs. Sanger Sequencing

	2X Bi-directional Sanger Sequencing Results					
Tumor Stage	**codon 12**	**codon 13**	**codon 61**	**wild-type**	**Total**	**% of Total**
Stage I	6	2	0	3	11	5.8%
Stage II	20	5	3	37	65	34.4%
Stage III	21	8	1	38	68	36.0%
Stage IV	17*	3*	3	20	43	22.8%
Unknown Stage	0	0	0	2	2	1.1%
Total	64	18	7	100	189	100.0%

* One of the 81 codon 12/13 mutant specimen contained mutations in both codon 12 and codon 13.

Results obtained when testing each of the 188 colorectal cancer tumor specimens with two lots of the **cobas** KRAS Test vs. results obtained using 2X bi-directional Sanger sequencing for KRAS exon 2, codon 12/13 mutations and exon 3, codon 61 mutations are shown in Tables 7 and 8.

Table 7
cobas KRAS Test, Lot 1 vs. 2X Bi-Directional Sanger Sequencing

		2X Bi-Directional Sanger Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 1	**MT Codon 12/13**	78	0	6	84
	MT Codon 61	0	6	1	7
	WT	2	1	93	96
	Totals	80	7	100	187*
Positive agreement = 96.6% (95% CI = 90.3 to 98.8%)					
Negative agreement = 93.0% (95% CI = 86.3 to 96.6%)					
Overall agreement = 94.7% (95% CI = 90.4 to 97.1%)					

MT: Mutant WT: Wild Type

*One specimen was not tested by Lot 1.

Table 8
cobas KRAS Test, Lot 2 vs. 2X Bi-Directional Sanger Sequencing

		2X Bi-Directional Sanger Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 2	**MT Codon 12/13**	79	0	6	85
	MT Codon 61	0	6	1	7
	WT	2	1	93	96
	Totals	81	7	100	188
Positive agreement = 96.6% (95% CI = 90.5 to 98.8%)					
Negative agreement = 93.0% (95% CI = 86.3 to 96.6%)					
Overall agreement = 94.7% (95% CI = 90.5 to 97.1%)					

MT: Mutant WT: Wild Type

Overall concordance of the **cobas** KRAS Test vs. 2X bi-directional Sanger sequencing for KRAS codon 12/13 and codon 61 mutations was 94.7% (10 total discordant results for each lot) for both lots of **cobas** KRAS Test reagents.

Discordant Testing by 454 Sequencing

Discordant results between the **cobas** KRAS Test vs. 2X bi-directional Sanger sequencing for Lot 1 and Lot 2 were resolved by 454 sequencing and are shown in Tables 9 and 10, respectively.

Table 9
cobas KRAS Test, Lot 1 vs. 2X-Bi-Directional Sanger Sequencing Resolved by 454 Sequencing

		2X Bi-Directional Sanger Sequencing, Resolved by 454 Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 1	**MT Codon 12/13**	83	0	1	84
	MT Codon 61	0	7	0	7
	WT	0	0	96	96
	Totals	83	7	97	187*
Positive agreement = 100.0% (95% CI = 95.9 to 100.0%)					
Negative agreement = 99.0% (95% CI = 94.4 to 99.8%)					
Overall agreement = 99.5% (95% CI = 97.0 to 99.9%)					

MT: Mutant WT: Wild Type

*One specimen was not tested by Lot 1.

Table 10
cobas KRAS Test, Lot 2 vs. 2X Bi-Directional Sanger Sequencing

		2X Bi-Directional Sanger Sequencing, Resolved by 454 Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 2	**MT Codon 12/13**	84	0	1	85
	MT Codon 61	0	7	0	7
	WT	0	0	96	96
	Totals	84	7	97	188
Positive agreement = 100.0% (95% CI = 95.9 to 100.0%)					
Negative agreement = 99.0% (95% CI = 94.4 to 99.8%)					
Overall agreement = 99.5% (95% CI = 97.0 to 99.9%)					

MT: Mutant WT: Wild Type

After discordant results between the **cobas** KRAS Test and 2X bi-directional Sanger sequencing were resolved by 454 sequencing, the overall concordance improved to 99.5% for both the first and the second lots of **cobas** KRAS Test reagents.

Specificity

Specificity of the **cobas** KRAS Test was determined by testing 188 colorectal cancer FFPET specimens in conjunction with the Correlation to a Reference Method study.

Sanger Sequencing

Specificity of the **cobas** KRAS Test was calculated by determining the percentage of FFPET specimens identified as KRAS wild-type by 2X bi-directional Sanger sequencing and correctly called KRAS wild-type by the **cobas** KRAS Test (negative percent agreement).

The specificity (negative percent agreement) obtained when testing the 188 tumor specimens for KRAS codon 12/13 and codon 61 mutations with the **cobas** KRAS Test vs. results obtained using 2X bi-directional Sanger sequencing was 93.0% (Tables 6 and 7) for both the first and the second lots of **cobas** KRAS Test reagents.

Discordant Testing by 454 Sequencing

The specificity improved to 99.0% for both the first and second lots (Tables 8 and 9) of **cobas** KRAS Test reagents after 454 sequencing was used to resolve discrepant results between the **cobas** KRAS Test and 2X bi-directional DNA Sanger sequencing when testing for KRAS codon 12/13 and codon 61 mutations.

The improvement in specificity of the **cobas** KRAS Test after 454 sequencing resolution was primarily due to the ability of 454 sequencing to detect KRAS mutations that were missed by Sanger sequencing due to the lower sensitivity of Sanger sequencing compared to 454 sequencing.

Cross-Reactivity

Cross-reactivity 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 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 exon 2, codon 12 silent mutations: GGA, GGC, and GGG; three KRAS exon 2, codon 13 silent mutations: GGA, GGT, and GGG. No cross-reactivity was observed with plasmids for KRAS exon 2, 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 or 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 5 KRAS codon 12, 13 and 61 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 (2 specimens), codon 13 (1 specimen) or codon 61 (2 specimens) mutations when 1 x 10^6 colony forming units were added during the tissue lysis step of a specimen containing a KRAS mutation at the levels listed in Table 11.

Table 11
Percent Mutation of Specimens Tested for Interference from Colon-Related Microorganisms

Specimen #	Mutation Status	% Mutation by 454 Sequencing
1	codon 12	15.9
2	codon 12	16.1
3	codon 13	42.8
4	codon 61	17.0
5	codon 61	19.8

Interference

Triglycerides (≤ 37mM, CLSI recommended high concentration[30]), hemoglobin (≤ 2 mg/mL, CLSI recommended high concentration[30]), and ≤ 50% necrotic tissue 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.

Robustness

Robustness of the **cobas** KRAS Test was determined using one KRAS exon 2, codon 12 mutant and one KRAS exon 3, codon 61 mutant colorectal cancer FFPET specimen at a mutation percentage of 13.1% and 17% mutation, respectively, Each specimen was sectioned into 100 five-µm sections for analysis.

Genomic DNA was extracted from each section using the **cobas**® DNA Sample Preparation Kit. A single replicate of the extracted genomic DNA was tested for each of the 100 sections for each of the 2 specimens. Ten separate runs were conducted with 20 sections analyzed per run with the **cobas** KRAS Test. One hundred (100) % of the replicates were reported as "Mutation Detected" by the **cobas** KRAS Test for both the KRAS codon 12 mutant specimen and the KRAS codon 61 mutant specimen yielding a false-negative rate of 0%.

Repeatability

Repeatability of the **cobas** KRAS Test was assessed using eight colorectal cancer (CRC) FFPET specimens. Characteristics of the specimens are outlined in Table 12.

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).

Table 12
cobas KRAS Test Repeatability

Specimen Number	KRAS Mutation Status	Mutation %	Mutation Detected (n=32)	Mutation Not Detected	Percent Agreement
1	Codon 12	16.4%	32	0	100%
2	Codon 12	20.6%	32	0	100%
3	Codon 13	16.7%	32	0	100%
4	Codon 13	10.3%	32	0	100%
5	Codon 61	28.1%	32	0	100%
6	Codon 61	31.5%	32	0	100%
7	Wild Type	-	0	32	100%
8	Wild Type	-	0	32	100%

The **cobas** KRAS Test had a correct call accuracy of 100% (256/256) for all days, specimens, replicates, operators and reagent lots combined.

II. NON-SMALL CELL LUNG CANCER (NSCLC) TISSUE

Analytical Sensitivity

The analytical sensitivity of the **cobas** KRAS Test was performed using dilution panels prepared from two types of specimens:

- Cell line blend prepared by mixing DNA stocks obtained from a KRAS mutant cell line and a KRAS wild-type cell line.
- Specimen blends prepared by mixing DNA stocks obtained from KRAS mutant FFPET specimens and KRAS wild-type FFPET specimens.

All specimens used in this study were sequenced by 454 sequencing in order to determine the percent mutation of each specimen.

Analytical Sensitivity of the cobas KRAS Test Using Cell Line Blends

DNA from lung cell lines containing either the KRAS codon 12 or 13 or 61 mutation were extracted and blended with DNA extracts from a KRAS wild-type cell line to achieve samples at 2.5%, 5%, and 10% mutation, verified by 454 sequencing. Three separate dilution panels contained the following dilutions (50.0, 12.5, 3.1, 0.8, and 0 ng/25 µL). Twenty-four (24) replicates of each panel member were tested, using each of 3 **cobas** KRAS Test kit lots (72 replicates total). Sensitivity was determined by the lowest amount of DNA that gave a KRAS "Mutation Detected" rate of at least 95%, shown in Table 13.

Table 13
Sensitivity of the cobas KRAS Test using Lung Cell Line Blend

KRAS Mutation	Specimen Type	Percent Mutation*	Amount of DNA in the Panel Member (ng/25 µL) to achieve ≥ 95% "Mutation Detected" Rate (N=72 replicates)
Codon 12 (Exon 2)	Cell-line	12.1%	0.8
		6.1%	3.1
		2.4%	50.0
Codon 13 (Exon 2)	Cell-line	7.8%	0.8
		4.9%	3.1
		2.8%	3.1
Codon 61 (Exon 3)	Cell-line	10.2%	0.8
		6.1%	0.8
		2.7%	12.5

* Mean percent mutation by 454 sequencing

The **cobas** KRAS Test gave a 95% "Mutation Detected" rate at 2.4% (at 50 ng/25 µL), 2.8% (at 3.1 ng/25 µL), 2.7% (at 12.5ng/25 µL) mutations for KRAS codon 12, 13, and 61 mutations, respectively. This demonstrates that the test will detect the KRAS mutations at 5% or greater with DNA input level of 50 ng/25 µL.

In addition, the **cobas** KRAS Test gave a 95% "Mutation Detected" rate at 3.1 ng/25 µL, 3.1 ng/25 µL, and 0.8 ng/25 µL (1:16, 1:16, and 1:64 dilutions of the recommended DNA input of 50 ng/25 µL) for KRAS codon 12, 13, and 61 mutations at ~5% mutation, respectively. This would indicate that the test will detect the KRAS mutations when ~94% of the DNA is degraded or non-amplifiable due to the fixation process, assuming that the cell line DNA blends contained 100% intact and amplifiable DNA.

Analytical Sensitivity Using FFPET Specimen blends

KRAS codon 12, 13 and 61 mutant FFPET specimen DNA extracts were blended with KRAS wild-type FFPET specimen extracts to achieve samples at ~5% mutation level. The final mutation levels for all specimens were verified by 454 sequencing. Each of the specimen blends was diluted to produce the panel members (50.0, 12.5, 3.1, 0.8, and 0 ng/25 µL).

Eight (8) replicates of each panel member were tested using each of 3 **cobas** KRAS Test kit lots (n=24/panel member). The sensitivity of each sample was determined by the lowest amount of DNA that gave a KRAS "Mutation Detected" rate of at least 95%, shown in Table 14.

Table 14
Sensitivity of the cobas KRAS Test using Lung Cancer Specimen Blends

KRAS Mutation	Specimen Type	Percent Mutation*	Amount of DNA in the Panel Member (ng/25 µL) to achieve ≥ 95% "Mutation Detected" Rate (N=24 replicates)
Codon 12 (Exon 2)	FFPET Blend	6.2%	12.5
		6.0%	3.1
		5.6%	3.1
Codon 13 (Exon 2)	FFPET Blend	6.2%	3.1
		4.4%	3.1
		4.4%	3.1
Codon 61 (Exon 3)	FFPET Blend	6.5%	3.1
		6.4%	3.1
		4.5%	3.1

* Mean percent mutation by 454 sequencing

This study demonstrates that the **cobas** KRAS Test can detect KRAS codon 12, 13 and 61 mutations at the ~5% mutation level using the standard input of 50 ng/25 µL. The ability of the test to detect the mutation at lower DNA input levels demonstrates that the specimens can contain degraded or non-amplifiable DNA from the fixation process and still be detected.

Correlation to Reference Method

One hundred and ninety-four (194) non-small cell lung cancer (NSCLC) FFPET specimens were tested using each of 2 lots of **cobas** KRAS Test kits. Comparison testing by 2X Bi-directional Sanger sequencing was performed on all specimens. Discordant results between the **cobas** KRAS Test and 2X Bi-directional Sanger sequencing were resolved using 454 sequencing.

cobas KRAS Test and 2X Bi-directional Sanger Sequencing Results

Specimen information and results of 2X bi-directional Sanger sequencing for the 194 specimens are summarized in Table 15. Ninety-five (95) of the 194 specimens were KRAS codon 12/13 mutant (one codon 12 mutant is also a mutant for codon 61) and one was KRAS codon 61 mutant, while 98 of the specimens were either KRAS wild-type or KRAS non-codon 12/13 mutant by Sanger sequencing and 192 specimens were either KRAS wild-type or KRAS non-codon 61 mutant.

Table 15
Tumor Stage vs. Sanger Sequencing

	2X Bi-directional Sanger Sequencing Results					
Tumor Stage	**codon 12**	**codon 13**	**codon 61**	**wild-type**	**Total**	**% of Total**
Stage I	15	2	0	29	46	23.7%
Stage II	12	0	0	7	19	9.8%
Stage III	21	4	1	9	35	18.0%
Stage IV	11	3	0	1	15	7.7%
Unknown Stage	24	3	0	52	79	40.7%
Total	83	12	1	98	194	100.0%

Results obtained when testing the 194 non-small cell lung cancer tumor specimens with the **cobas** KRAS Test vs. results obtained using 2X bi-directional Sanger sequencing for KRAS exon 2, codon 12/13 mutations and exon 3, codon 61 mutations are shown in Tables 16 and 17.

Table 16
cobas KRAS Test, Lot 1 vs. 2X Bi-Directional Sanger Sequencing

		2X Bi-Directional Sanger Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 1	**MT Codon 12/13**	90	0	6	96
	MT Codon 61	0	1	1	2
	WT	5	1	91	97
	Totals	95	2	98	195*
Positive agreement = 93.8% (95% CI = 87.2% to 97.1%)					
Negative agreement = 92.9% (95% CI = 86.0% to 96.5%)					
Overall agreement = 93.3% (95% CI = 88.9% to 96.1%)					

MT: Mutant WT: Wild Type

* N=194, one specimen is a double mutant (codon 12 and codon 61) by Sanger

Overall concordance of the **cobas** KRAS Test vs. 2X bi-directional Sanger sequencing for KRAS codon 12/13 and 61 mutations was 93.8% (a total of 13 discordant results) for lot 1 of **cobas** KRAS Test reagents.

Table 17
cobas KRAS Test, Lot 2 vs. 2X Bi-Directional Sanger Sequencing

		2X Bi-Directional Sanger Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 2	**MT Codon 12/13**	90	0	8	98
	MT Codon 61	0	1	1	2
	WT	5	1	88	94
	Totals	95	2	97	194*
Positive agreement = 93.8% (95% CI = 87.2% to 97.1%)					
Negative agreement = 90.7% (95% CI = 83.0% to 95.0%)					
Overall agreement = 92.3% (95% CI = 87.6% to 95.3%)					

MT: Mutant WT: Wild Type

* N=193, one specimen is a double mutant (codon 12 and codon 61) by Sanger. One of the 194 specimens gave invalid result.

Overall concordance of the **cobas** KRAS Test vs. 2X bi-directional Sanger sequencing for KRAS codon 12/13 and 61 mutations was 93.8% (a total of 15 discordant results) for lot 2 of **cobas** KRAS Test reagents.

Discordant Testing by 454 Sequencing

Discordant results between the **cobas** KRAS Test vs. 2X bi-directional Sanger sequencing for KRAS codon 12/13 and codon 61 were resolved by 454 sequencing and are shown in Tables 18 and 19.

Table 18
cobas KRAS Test, Lot 1 vs. 2X-Bi-Directional Sanger Sequencing Resolved by 454 Sequencing

		Sanger Sequencing, Resolved with 454 Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 1	**MT Codon 12/13**	96	0	0	96
	MT Codon 61	0	1	1	2
	WT	3	0	94	97
	Totals	99	1	95	195*
Positive agreement = 97.0% (95% CI = 91.5% to 99.0%)					
Negative agreement = 98.9% (95% CI = 94.3% to 99.8%)					
Overall agreement = 97.9% (95% CI = 94.8% to 99.2%)					

MT: Mutant WT: Wild Type

*N=194, one specimen is a double mutant (codon 12 and codon 61) by Sanger

Table 19
cobas KRAS Test, Lot 2 vs. 2X-Bi-Directional Sanger Sequencing Resolved by 454 Sequencing

		Sanger Sequencing, Resolved with 454 Sequencing			
		MT Codon 12/13	**MT Codon 61**	**WT**	**Totals**
cobas KRAS Lot 2	**MT Codon 12/13**	98	0	0	98
	MT Codon 61	0	1	1	2
	WT	0	0	94	94
	Totals	98	1	95	194*
Positive agreement = 100.0% (95% CI = 96.3% to 100.0%)					
Negative agreement = 98.9% (95% CI = 94.3% to 99.8%)					
Overall agreement = 99.5% (95% CI = 97.1% to 99.9%)					

MT: Mutant WT: Wild Type

*N=193, one specimen is a double mutant (codon 12 and codon 61) by Sanger. One of the 194 specimens gave invalid result.

After discordant results between the **cobas** KRAS Test and 2X bi-directional Sanger sequencing were resolved by 454 sequencing, the overall concordance improved to 97.9% and 99.5% for Lot 1 and Lot 2 of **cobas** KRAS Test reagents, respectively.

Specificity

Specificity of the **cobas** KRAS Test was determined by testing 194 NSCLC FFPET specimens in conjunction with the Correlation to a Reference Method study.

Sanger Sequencing

Specificity of the **cobas** KRAS Test was calculated by determining the percentage of FFPET specimens identified as KRAS wild-type by 2X bi-directional Sanger sequencing and correctly called KRAS wild-type by the **cobas** KRAS Test (negative percent agreement).

The specificity (negative percent agreement) obtained when testing the 194 tumor specimens for KRAS codon 12/13 and codon 61 mutations with the **cobas** KRAS Test vs. results obtained using 2X bi-directional Sanger sequencing was 92.9% (Table 15) and 90.7% (Table 16) for Lot 1 and Lot 2 of **cobas** KRAS Test reagents, respectively.

Discordant Testing by 454 Sequencing

The specificity improved to 98.9% for both Lots (Tables 18 and 19) of **cobas** KRAS Test reagents after 454 sequencing was used to resolve discrepant results between the **cobas** KRAS Test and 2X bi-directional DNA Sanger sequencing when testing for KRAS codon 12/13 and codon 61 mutations.

The improvement in specificity of the **cobas** KRAS Test after 454 sequencing resolution was primarily due to the ability of 454 sequencing to detect KRAS mutations that were missed by Sanger sequencing due to the lower sensitivity of Sanger sequencing compared to 454 sequencing.

Cross-Reactivity with Lung-related Microorganisms

Cross-reactivity of the **cobas** KRAS Test was evaluated by testing lung-related microorganisms.

Cross-reactivity was also evaluated by determining whether the presence of microorganisms interfered with detection of KRAS codon 12, 13, and 61 mutations.

The following lung-related microorganisms were found not to cross react in the **cobas** KRAS Test when added to 5 KRAS codon 12, 13 and 61 wild-type specimens at 1 x 10^6 colony forming units during the tissue lysis step:

1. *Streptococcus pneumoniae*
2. *Haemophilus influenzae*

The tested microorganisms also did not interfere with the detection of KRAS codon 12 (3 specimens), codon 13 (1 specimen) or codon 61 (1 specimen) mutations when 1 x 10^6 colony forming units were added during the tissue lysis step of a specimen containing a KRAS mutation at the levels listed in Table 20.

Table 20
Percent Mutation of Specimens Tested for Interference from Lung-Related Microorganisms

Specimen #	Mutation Status	% Mutation by 454 Sequencing
1	codon 12	15.3
2	codon 12	32.3
3	codon 12	32.5
4	codon 13	13.4
5	codon 61	32.6

Interference

Triglycerides (≤ 37mM, CLSI recommended high concentration[30]), hemoglobin (≤ 2 mg/mL, CLSI recommended high concentration[30]), and ≤ 80% necrotic tissue 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.

Robustness

Robustness of the **cobas** KRAS Test was determined using FFPET specimens from NSCLC with KRAS mutations in codon 12, 13 and 61.

Genomic DNA was extracted from each section using the **cobas**® DNA Sample Preparation Kit. A single replicate of the extracted genomic DNA was tested for 100 sections of a codon 12 specimen, 102 sections of a codon 13 specimen, and 100 sections of a codon 61 specimen. Fourteen separate runs were conducted with the **cobas** KRAS Test. All one-hundred replicates of each codon 12 and codon 61 specimens were reported as "Mutation Detected". One-hundred of the codon 13 specimen were reported as "Mutation Detected", one replicate was reported as "Mutation Not Detected", and one replicate was reported as "Invalid" by the **cobas** KRAS Test. The overall false-negative rate of is 0.7% (2/302).

Repeatability

Repeatability of the **cobas** KRAS Test was assessed using eight non-small cell lung cancer FFPET specimens. Characteristics of the specimens are outlined in Table 21.

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).

Table 21
cobas KRAS Test Repeatability

Specimen Number	KRAS Mutation Status	Mutation %	Mutation Detected (n=32)	Mutation Not Detected	Percent Agreement
1	Codon 12	15.3%	32	0	100%
2	Codon 12	17.5%	32	0	100%
3	Codon 13	13.3%	32	0	100%
4	Codon 13	27.8%	32	0	100%
5	Codon 61	14.1%	32	0	100%
6	Codon 61	32.6%	32	0	100%
7	Wild Type	-	0	32	100%
8	Wild Type	-	0	32	100%

The **cobas** KRAS Test had a correct call accuracy of 100% (256/256) for all days, specimens, replicates, operators and reagent lots combined.

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 22.

Table 23 lists all result flags of the system that are user relevant.

Table 22 Flag Source

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

Table 23 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.
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.

Flag Code	Severity	Description	Recommended Action
			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.
R259, R269	Error	Calibrator out of range.	Repeat the run. Refer to the *Quality Control* section of the test-specific instructions for use.

Flag Code	Severity	Description	Recommended Action
			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).
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.

Flag Code	Severity	Description	Recommended Action
			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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Document Revision Information	
Doc Rev. 11.0 06/2020	Removed the following DNA Sample Preparation kit information: • Reagent listings and composition information. • Related procedural steps and notes. Added reference to the **cobas**® DNA Sample Preparation Kit Instructions for Use at the beginning and in the Procedural Limitations section. Added a new Procedural Limitation regarding invalid re-testing. Updated System and Operator Manual references throughout to "**cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance". Added results flags. Made corrections to typos and updated for consistency and standardization of language throughout. Updated International Air Transport Association reference. Added statement to clarify that product safety labeling primarily follows EU GHS guidance. Updated the harmonized symbol page. Updated distributors addresses and trademarks and patents section. Please contact your local Roche Representative if you have any questions.

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The following symbols are used in labeling for Roche PCR diagnostic products.

	Ancillary Software		*In vitro* diagnostic medical device
	Authorized representative in the European Community		For IVD performance evaluation only
	Barcode Data Sheet		Lower Limit of Assigned Range
	Batch code		Manufacturer
	Biological risks		Store in the dark
	Catalogue number		Temperature limit
	Consult instructions for use		Test Definition File
	Contains sufficient for <*n*> tests		Upper Limit of Assigned Range
	Contents of kit		Use-by date
	Distributed by		Global Trade Item Number
Rx Only	US Only: Federal law restricts this device to sale by or on the order of a physician.		Date of manufacture

 CE marking of conformity; this device is in conformity with the applicable requirements for CE marking of an in vitro diagnostic medical device

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