cobas® 4800 BRAF V600 Mutation Test

FOR *IN VITRO* DIAGNOSTIC USE.

cobas® 4800 BRAF V600 Mutation Test | **BRAF** | 24 Tests | M/N: 05985595190

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

INTENDED USE

The primary use of the **cobas**® 4800 BRAF V600 Mutation Test is the detection of the BRAF V600 mutations in DNA extracted from formalin-fixed, paraffin-embedded human melanoma and papillary thyroid carcinoma (PTC) tissue. In melanoma, it is intended to be used as an aid in selecting patients whose tumors carry BRAF V600 mutations for treatment either with ZELBORAF® (vemurafenib) alone, or with COTELLIC® (cobimetinib) in combination with ZELBORAF® (vemurafenib).

SUMMARY AND EXPLANATION OF THE TEST

Activating mutations of the proto-oncogene BRAF occur in many human cancers, including malignant melanoma, colorectal cancer, ovarian cancer, and thyroid cancer.[1, 2] BRAF mutations have been identified in 40%-60% of malignant melanomas[3] and in 36-46% of papillary thyroid carcinomas.[4, 5] BRAF mutations are also common in benign nevi,[6] suggesting that such mutations are a very early event. The discovery of such somatic mutations in the BRAF gene in melanoma, PTC, and other human tumors has helped to elucidate the central role of the BRAF kinase in signaling pathways that control cellular proliferation, differentiation and cell death. In normal cells, BRAF is part of a highly regulated signaling pathway that mediates the effects of growth factor receptors (such as EGFR) through RAS, RAF, MEK and ERK. Oncogenic mutations in BRAF result in a gain of kinase function, rendering the RAF-MEK-ERK pathway constitutively active in the absence of the typical growth factors.

The majority of BRAF mutations in melanoma, PTC, and other human tumors occur in codon 600.[7] The predominant mutation at codon 600 is the V600E mutation (GTG > GAG). A number of dinucleotide mutations affecting codon 600 (V600K, [(GTG > AAG), V600R (GTG > AGG) V600E2 (GTG > GAA) and V600D (GTG > GAT)] have also been observed less commonly, primarily in melanoma and rarely in other tumors, such as colorectal cancer.

The **cobas**® 4800 BRAF V600 Mutation Test is a real-time PCR assay designed to detect the presence of the V600E (T1799A) mutation. The **cobas**® 4800 BRAF V600 Mutation Test is used as a companion diagnostic test for vemurafenib, a compound which inhibits the mutant V600E version of BRAF. Clinical trials of vemurafenib in patients with advanced melanoma have shown that patients with a V600E-mutant tumor are likely to experience clinical benefit from the compound.[8, 9] Subsequently, a clinical trial of cobimentinib, in combination with vemurafenib, in patients with advanced melanoma has shown that patients with either a V600E- or V600K-mutant tumor that is detected by the **cobas**® 4800 BRAF V600 Mutation Test are likely to experience clinical benefit from this therapy.[10, 11] V600K is present in approximately 10-15% of melanoma specimens with BRAF V600 mutations.[12]

PRINCIPLES OF THE PROCEDURE

The **cobas**® 4800 BRAF V600 Mutation Test (**cobas** BRAF Test) is based on two processes: (1) manual specimen preparation to obtain genomic DNA from formalin-fixed, paraffin-embedded tissue (FFPET); (2) PCR amplification and detection of target DNA using a complementary primer pair and two oligonucleotide probes labeled with different fluorescent dyes. One probe is designed to detect the wild-type BRAF V600 sequence and one is designed to detect the V600E mutation sequence. Two external run controls are provided and the wild-type allele serves as an internal, full process control.

Specimen Preparation

FFPET specimens are processed and genomic DNA isolated using the **cobas**® DNA Sample Preparation Kit, a 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 and 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** BRAF Test kit.

PCR Amplification and Detection

Target Selection

The **cobas** BRAF Test uses primers that define a 116-base pair sequence of human genomic DNA containing the BRAF codon 600 site in exon 15. The entire BRAF gene is not amplified. The **cobas** BRAF Test is designed to detect the nucleotide 1799 T > A change in the BRAF gene which results in a valine-to-glutamic acid substitution at codon 600 (V600E). BRAF wild-type and mutant DNA target-specific fluorescent dye-labeled TaqMan probes bind to the wild-type and mutant sequences respectively. The wild-type and mutant sequences are detected by using a dedicated optical channel for each sequence.

Target Amplification

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 dNTPs, extends each annealed primer, thus synthesizing a second DNA strand. This completes the first cycle of PCR, yielding a double-stranded DNA copy of the targeted 116-basepair region of the BRAF 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 region of the BRAF gene between the primers.

Automated Real-time Detection

The **cobas** BRAF Test utilizes real-time PCR technology. Each target-specific, oligonucleotide probe in the reaction is labeled with a fluorescent dye that serves as a reporter, and with a quencher molecule that absorbs (quenches) fluorescent emissions from the reporter dye within an intact probe. During each cycle of amplification, probe complementary to the single-stranded DNA sequence in the amplicon binds and is subsequently cleaved by the 5' to 3' nuclease activity of the Z05 DNA Polymerase. Once the reporter dye is separated from the quencher by this nuclease activity, fluorescence of a characteristic wavelength can be measured when the reporter dye is excited by the appropriate spectrum of light. Two different reporter dyes are used to label the target-specific BRAF wild-type (WT) probe and the BRAF V600E mutation probe. Amplification of the two BRAF sequences can be detected independently in a single reaction well by measuring fluorescence at the two characteristic wavelengths in dedicated optical channels.

Selective Amplification

Selective amplification of target nucleic acid from the specimen is achieved in the **cobas** BRAF Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP).[13] 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 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 55ºC, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon.

REAGENTS

cobas® 4800 BRAF V600 Mutation Test (BRAF) **24 Tests** (M/N: 05985595190)			
Kit components	**Reagent ingredients**	**Quantity per kit**	**Safety symbol and warning**[a]
RXNMIX **(Reaction Mix)**	Tricine buffer Potassium acetate Potassium hydroxide Glycerol Tween 20 EDTA 5% Dimethyl sulfoxide < 0.09% dNTPs < 0.10% Z05 DNA polymerase (microbial) < 0.10% AmpErase (uracil-N-glycosylase) enzyme (microbial) <0.003% Oligonucleotide aptamer 0.08% Sodium azide	3 x 0.16 mL	N/A
MGAC **(Magnesium acetate)**	Magnesium Acetate 0.09% Sodium azide	3 x 0.15 mL	N/A
BRAF OM **(BRAF Oligo Mix)**	Tris-HCl buffer EDTA 0.09% Sodium azide Poly rA RNA (synthetic) < 0.01% Upstream and downstream BRAF primers < 0.01% Fluorescent-labeled BRAF probes	3 x 0.13 mL	N/A
BRAF MUT **(BRAF Mutant Control)**	Tris-HCl buffer EDTA Poly rA RNA (synthetic) 0.05% Sodium azide < 0.001% plasmid DNA (microbial) containing BRAF mutant sequence < 0.001% plasmid DNA (microbial) containing BRAF wild-type sequence	2 x 0.13 mL	N/A
BRAF WT **(BRAF Wild-Type Control)**	Tris-HCl buffer EDTA Poly rA RNA (synthetic) 0.05% Sodium azide < 0.001% plasmid DNA (microbial) containing BRAF wild-type sequence	2 x 0.13 mL	N/A
DNA SD **(DNA Specimen Diluent)**	Tris-HCl buffer 0.09% Sodium azide	2 x 1 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 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 **cobas**® 4800 reagents to prevent contamination.

K. To avoid contamination of the working master mix 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 master mix 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*[14] and in the CLSI Document M29 A4.[15]

M. **RXNMIX**, **MGAC**, **BRAF MUT, BRAF WT,** 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 single 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 pipets and DNase-free pipet tips is recommended.

STORAGE AND HANDLING REQUIREMENTS

A. Do not freeze reagents.

B. Store **RXNMIX**, **MGAC**, **BRAF OM**, **BRAF MUT**, **BRAF WT**, and **DNA SD** at 2-8°C. Once opened, these reagents are stable for up to 4 uses over 60 days or until the expiration date, whichever comes first.

C. **BRAF OM** and Working Master Mix (prepared by the addition of **BRAF OM** and **MGAC** to **RXNMIX**) should be protected from prolonged exposure to light.

D. Working Master Mix (prepared by the addition of **BRAF OM** and **MGAC** to **RXNMIX**) must be stored at 2-8ºC in the dark. The prepared specimens and controls must be added within 1 hour of preparation of the Working Master Mix.

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 Master Mix (prepared by the addition of **BRAF OM** and **MGAC** to **RXNMIX**).

MATERIALS PROVIDED

cobas® 4800 BRAF V600 Mutation Test (M/N: 05985595190) **BRAF** **24 Tests**

RXNMIX
(Reaction Mix) (Cap with Natural Button)

MGAC
(Magnesium acetate) (Cap with Yellow Button)

BRAF OM
(BRAF Oligo Mix) (Black Cap with White Button)

BRAF MUT
(BRAF Mutant Control) (Cap with Red Button)

BRAF WT
(BRAF Wild-Type Control) (Cap with Blue Button)

DNA SD
(DNA Specimen Diluent) (Cap with Purple Button)

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)
- 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
- 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 the 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
- BRAF Analysis Package Software version 1.0 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

FFPET specimens have been validated for use with the **cobas** BRAF Test.

B. Specimen Transport

FFPET specimens can be transported at 15-30ºC. Transportation of FFPET specimens must comply with country, federal, state and local regulations for the transport of etiologic agents.[16]

C. Specimen Storage

Stability of FFPET specimens stored at 15-30°C for up to 12 months after the date of collection has been confirmed. FFPET 5-µm sections mounted on slides may be stored at 15-30°C for up to 60 days.

INSTRUCTIONS FOR USE

NOTE: All reagents except RXNMIX, MGAC, and BRAF OM must be at ambient temperature prior to use. The RXN MIX, MGAC, and BRAF OM may be taken directly from 2-8°C storage to prepare Working Master Mix.

NOTE: Only melanoma and PTC FFPET sections of 5 µm thickness containing at least 50% tumor cells are to be used in the cobas BRAF Test. Any specimen containing less than 50% tumor cells should be macro-dissected after deparaffinization.

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

The **cobas** BRAF Test kit is designed to run from a minimum of 3 specimens plus controls up to a maximum of 24 specimens plus controls. Fewer than 3 specimens plus controls can be run at one time, but may result in insufficient volume of reagents to run a total of 24 specimens plus controls with the kit. The **cobas** BRAF Test contains reagents sufficient for 8 runs of 3 specimens plus controls. One replicate of the **cobas** BRAF Test Mutant Control [**BRAF MUT**] and one replicate of the **cobas** BRAF Test Wild-Type Control [**BRAF WT**] are required to perform each run (see *"Quality Control"* section).

Workflow

NOTE: The cobas BRAF Test can be used for up to 24 specimens in a run.

NOTE: To maximize reagent use, a test run should include a minimum of three (3) patient specimens plus controls.

DNA Isolation

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

Macrodissection

If the sample contains less than 50% 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 before quantitation.

B. Quantify DNA using a spectrophotometer according to the manufacturer's protocol. Use **DNA EB** provided in the **cobas**® DNA Sample Preparation Kit as the blank for the instrument. An average of 2 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 ± 2.0 ng/µL.

C. DNA stock concentration must be ≥ 5 ng/µL to perform the **cobas** BRAF Test.

NOTE: Each DNA stock must have a minimum concentration of 5 ng/µL to perform the cobas BRAF Test. If the concentration of a DNA stock is < 5 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 and PK from the cobas® DNA Sample Preparation Kit and perform DNA isolation and quantitation. For unmounted samples, combine two the tissue from both sections into one tube and perform deparaffinization, DNA isolation and quantitation. If the DNA stock is still < 5 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 the working master mix 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 master mix 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 analyzer set-up.

Test Order Set-Up:

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

Dilution Calculation of Specimen DNA Stock:

Only one amplification/detection is run per specimen, using 25 µL of a 5 ng/µL dilution of DNA stock (125 ng in total). The instructions below describe how to prepare a minimum of 35 µL of diluted DNA stock at 5 ng/µL, dependent on the initial DNA stock concentration. This will ensure each specimen uses a minimum of 5 µL of DNA stock to prevent variation that may occur when pipetting smaller volumes of sample.

Dilution Calculation of Specimen DNA Stock at Concentrations from 5 ng/µL to 35 ng/µL

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

NOTE: Only one amplification/detection is run per specimen, using 25 µL of a 5 ng/µL dilution of DNA stock (125 ng in total).

A. For each specimen, determine the amount of DNA stock required using the following formula:
   Volume of DNA stock required = (35 µL x 5 ng/µL) / DNA stock concentration in ng/µL
B. For each specimen, determine the amount of DNA Specimen Diluent (**DNA SD**) required using the following formula:
   Volume of DNA SD required in µL = (35 µL - Volume of DNA stock required in µL).
   Example:
   DNA stock concentration = 21 ng/µL
   A. Volume of DNA stock required = (35 µL x 5 ng/µL) / 21 ng/µL = 8.3 µL
   B. Volume of DNA SD required in µL = (35 µL – 8.3 µL) = 26.7 µL

Dilution Calculation of Specimen DNA Stock at Concentrations >35 ng/µL

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

NOTE: Only one amplification/detection is run per specimen, using 25 µL of a 5 ng/µL dilution of DNA stock (125 ng in total).

A. At DNA stock concentrations > 35 ng/µL, use the following formula to calculate the amount of DNA Specimen Diluent (**DNA SD**) required to prepare at least 35 µL of diluted DNA stock. This is to ensure each specimen uses a minimum of 5 µL of DNA stock,
   Vol. of DNA SD required in µL = ((5 µL DNA stock x DNA stock conc. in ng/µL) / (5ng/µL)) – 5 µL
B. Use the calculated volume of **DNA SD** to dilute 5 µL of DNA stock
   Example:
   DNA stock concentration = 42 ng/µL
   A. Vol. of DNA SD required in µL = ((5 µL x 42 ng/µL) / (5 ng/µL)) – 5 µL = 37 µL
   B. Use the calculated volume of **DNA SD** to dilute 5 µL of DNA stock.

Specimen Dilution

A. Prepare the appropriate number of 1.5 mL microcentrifuge tubes for specimen DNA stock dilutions by labeling them with the proper specimen identification in the specimen addition area.
B. Using a pipettor with an aerosol-resistant pipette tip, pipette the calculated volume of DNA Specimen Diluent (**DNA SD**) into each labeled specimen tube.
C. Vortex each specimen DNA stock for 10 seconds.
D. Using a pipettor with an aerosol-resistant pipette tip, gently pipette the calculated volume of each specimen DNA stock into the appropriately labeled tube containing **DNA SD**. Use a new pipette tip for each specimen.
E. Cap and mix each diluted specimen DNA stock by vortexing 10 seconds.
F. Change gloves.

Preparation of Working Master Mix (MMX)

NOTE: The BRAF Oligo Mix and working MMX are light-sensitive. All open mixtures of BRAF OM and working MMX should be protected from prolonged exposure to light.

A. Calculate the volume of **RXNMIX** required using the following formula:

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

B. Calculate the volume of **BRAF OM** required using the following formula:

Volume of **BRAF OM** required = (Number of Specimens + 2 Controls + 1) x 8 µL

C. Calculate the volume of **MGAC** required using the following formula:

Volume of **MGAC** required = (Number of Specimens + 2 Controls + 1) x 7 µL

Table 1 may be used to determine volumes 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
# of Specimens*

		1	2	3	4	5	6	7	8	9	10
RXN MIX	**10 µL**	40	50	60	70	80	90	100	110	120	130
BRAF OM	**8 µL**	32	40	48	56	64	72	80	88	96	104
MGAC	**7 µL**	28	35	42	49	56	63	70	77	84	91
Total Vol. µL		**100**	**125**	**150**	**175**	**200**	**225**	**250**	**275**	**300**	**325**

* # of Specimens + 2 Controls + 1

D. Remove appropriate number of **RXNMIX, BRAF OM** and **MGAC** vials from 2°-8°C storage. Vortex each reagent for 5 seconds to collect liquid at the bottom of the tube before use. Label a sterile microcentrifuge tube for the working master mix (MMX).

E. Add the calculated volume of **RXNMIX** to the labeled MMX tube.

F. Add the calculated volume of **BRAF OM** to the labeled MMX tube.

G. Add the calculated volume of **MGAC** to the labeled MMX tube.

H. Vortex tube for 5 seconds to assure adequate mixing.

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

I. Carefully add 25 µL of working MMX to each reaction well of the AD-plate that is needed for the run. Do not allow the pipette tip to touch the plate outside that well.

Addition of Controls and Specimens:

A. Add 25 µL of **BRAF MUT** Control to well **A01** of the AD-plate and mix well using pipettor to aspirate and dispense within the well a minimum of two times.

B. Using a new pipette tip, add 25 µL of **BRAF WT** Control to well **B01** of the AD-plate and mix well using pipettor to aspirate and dispense within the well a minimum of two times.

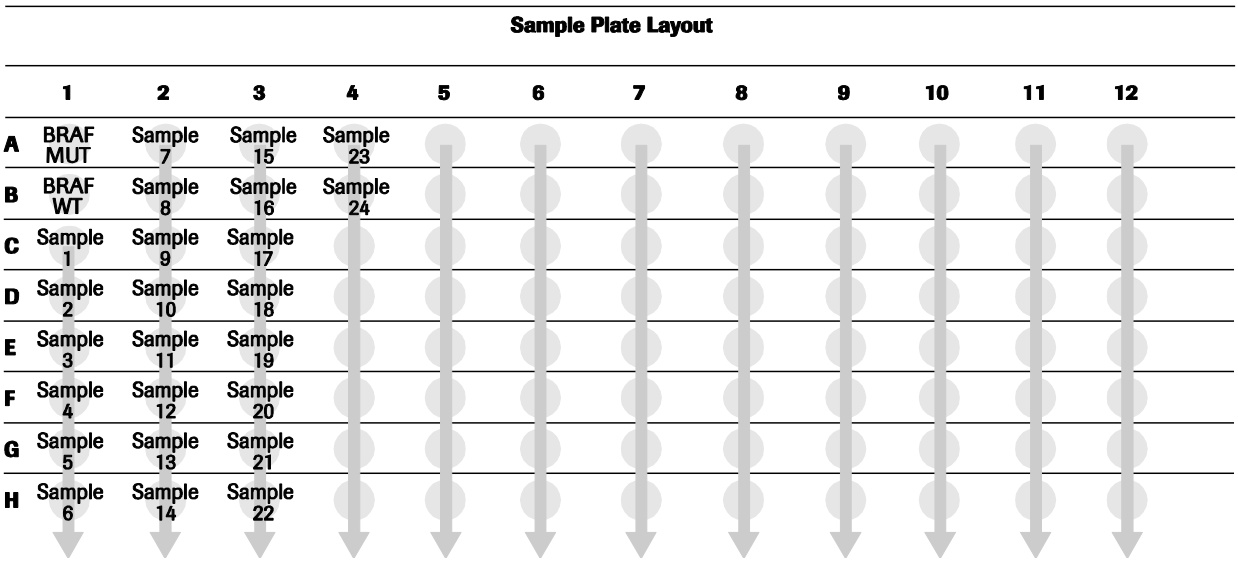
NOTE: Each run must contain both a BRAF MUT Control in position A01 and a BRAF WT Control in position B01 or the run will be invalidated by the cobas z 480 analyzer.

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

C. Using a pipettor with an aerosol-resistant tip, add 25 µL of diluted specimen DNA to the appropriate well containing working MMX, starting from position **C01** on the AD-plate following the template in Figure 1 below. Mix the reaction by using the pipettor to aspirate and dispense within the well a minimum of two times. Ensure that all liquid is collected at the bottom of the well.

NOTE: Specimen DNA and Controls should be added to the AD-plate within 1 hour after the preparation of the working Master Mix (MMX).

Figure 1



D. Continue until all test specimens have been added to the AD-plate.

E. Cover the AD-plate with the sealing film (supplied with the plates). Use the sealing film applicator to ensure that the sealing film adheres firmly to the AD-plate.

F. Confirm that all liquid is collected at the bottom of each well before starting Amplification and Detection.

NOTE: Amplification and detection should be started within 1 hour after the addition of Specimen DNA and Controls to the working MMX.

Starting PCR

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

INTERPRETATION OF RESULTS

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

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

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

Table 2
Interpretation of Specimen Results

cobas BRAF Test Result	*Interpretation*
Mutation Detected	V600 Mutation Detected in the BRAF Codon 600 site in exon 15
Mutation Not Detected or No Mutation Detected*	V600 Mutation Not Detected in the BRAF Codon 600 site in exon 15
Invalid	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	Failed run due to hardware or software failure

** A Mutation Not Detected or No Mutation Detected result does not preclude the presence of a mutation in the BRAF Codon 600 site because results depend on the number of mutant sequence copies present in the specimen and may be affected by specimen integrity, amount of isolated DNA, and the presence of interfering substances.*

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.

Note: If there is not enough specimen DNA stock remaining to perform a new dilution of the DNA stock, obtain a new 5-µm section of tissue and re-isolate DNA using the cobas® DNA Sample Preparation Kit (M/N 05985536190), then proceed with Step B below.

B. After performing the DNA stock dilution to 5 ng/µL described in "**Specimen Dilution**", perform an additional 1:2 dilution by taking 20 µL of the diluted DNA stock and adding 20 µL of DNA Specimen Diluent (**DNA SD**).

C. Continue with "**Preparation of Working Master Mix (MMX)**" and the remainder of the amplification and detection procedure.

Note: If the specimen remains invalid after retesting at a 1:2 dilution, repeat the entire test procedure for that specimen, starting with DNA isolation using the cobas® DNA Sample Preparation Kit (M/N 05985536190) using a new 5-µm FFPET section. The standard 25 µL of DNA at 5 ng/µL (without further dilution) should be used for amplification and detection.

QUALITY CONTROL

The **cobas** BRAF Test Mutant (**BRAF MUT**) Control and Wild-Type (**BRAF WT**) Control are included in each run. A run is valid if both the **BRAF MUT** Control well (**A01**) and the **BRAF WT** Control well (**B01**) have a valid control status. If either the **BRAF MUT** Control or **BRAF WT** Control is invalid, the run 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.

BRAF Mutant Control

The **BRAF MUT** Control result must be 'Valid'. If the **BRAF MUT** Control results are consistently invalid, contact your local Roche office for technical assistance.

BRAF Wild-Type Control

The **BRAF WT** Control result must be 'Valid'. If the **BRAF WT** Control 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 PCR-based tests, care should be taken to keep reagents and amplification mixtures free of contamination.

PROCEDURAL LIMITATIONS

1. Test only the indicated specimen types. The **cobas** BRAF Test has been validated for use only with melanoma and PTC FFPET specimens.
2. The **cobas** BRAF Test has been validated using only the **cobas**® DNA Sample Preparation Kit (Roche M/N: 05985536190) to extract genomic DNA.
3. Detection of a mutation is dependent on the number of mutant sequence 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 this Package Insert and in the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance .
5. The addition of AmpErase enzyme into the **cobas** BRAF Test Reaction Mix enables selective amplification of target DNA; however, good laboratory practices and careful adherence to the procedures specified in this Package Insert 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**® 4800 System has been validated for use with this product. No other PCR System has been validated 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 and variants within the regions of the BRAF gene covered by the primers or probes used in the **cobas** BRAF Test may result in failure to amplify the BRAF V600 allele or detect the presence of mutation in codon 600.
11. The presence of PCR inhibitors may cause false negative or invalid results.
12. Melanin is a known inhibitor of PCR reactions. The DNA sample preparation kit removes melanin from the specimen during extraction; however, melanin in a specimen may still cause invalid results. If melanin inhibition is suspected, repeat testing using a 1:2 dilution is suggested, as described in "Retesting of Specimens with Invalid Results."
13. The **cobas** 4800 BRAF V600 Mutation Test shows limited cross-reactivity with non-V600E mutant specimens (V600K, V600D, and V600E2). Refer to the Melanoma Non-clinical Performance Evaluation section for more details.
14. FFPET specimens containing degraded DNA may affect the ability of the test to detect the mutation.
15. The **cobas** 4800 BRAF Mutation Test is a qualitative test. The test is not for quantitative measurements of mutation.

I. MELANOMA

NON-CLINICAL PERFORMANCE EVALUATION

For the nonclinical studies described below, % tumor content was assessed by pathology review and melanin content was assessed by pathology review and an in-house melanin determination assay. Bi-directional Sanger sequencing was used to select the specimens for testing. The % mutation level was determined using 454 sequencing (quantitative massively-parallel pyrosequencing method).

Analytical Sensitivity

Analytical Sensitivity- Limit of Detection (LoD)

The minimum amount of input DNA that produces correct results 95% of the time was assessed using dilution panels prepared from three types of specimens:

- Specimen blends prepared by mixing DNA stocks obtained from BRAF V600E mutant FFPET specimens and BRAF wild-type FFPET specimens to achieve specific mutation levels.
- Individual FFPET DNA stocks prepared from three BRAF V600E mutant FFPET specimens.
- Cell line blend prepared by mixing DNA stocks obtained from a BRAF V600E mutant cell line and a BRAF wild-type cell line.

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

Analytical Sensitivity Using Specimen blends

BRAF V600E mutant FFPET specimen DNA stocks were blended with BRAF wild-type FFPET specimen DNA stocks to achieve one specimen at ~10%, three specimens at ~5%, and one specimen at ~3% mutation level. One BRAF wild-type specimen was also tested. After blending, the mutation levels were verified by 454 sequencing. Each of the five specimen blends with V600E mutation (but not the wild-type specimen) was then diluted to produce the panel members detailed in Table 3.

Table 3
Preparation of Dilution Panel Members from Specimen Blends

Blend	Mean % Mutation*	Amount of DNA in Dilution Panel Members (ng/25 µL)**
10% Blend	9% (n = 6)	125, 62.5, 31.3
5% Blend 1	5% (n = 5)	125, 5, 2.5, 1.3, 0.6, 0.3
5% Blend 2	5% (n = 5)	125, 5, 2.5, 1.3, 0.6, 0.3
5% Blend 3	6% (n = 5)	125, 5, 2.5, 1.3, 0.6, 0.3
2.5% Blend	3% (n = 5)	125, 62.5, 31.3
0% (Wild-type only)	- - -	125

* *Mean percent mutation of the blend, tested by 454 sequencing*
** *Amount of genomic DNA contained in each panel member. 25 µL is the sample input volume for the test.*

Eight (8) replicates of each panel member were run using each of 3 **cobas** BRAF Test kit lots (n=24/panel member). Table 4 shows the sensitivity of each FFPET blend, determined by the lowest amount of DNA that gave a BRAF V600E "Mutation Detected" rate of at least 95% (shaded rows).

Table 4
Sensitivity of the cobas BRAF Test using FFPET Blends

FFPET Blend	Percent Mutation by 454 Sequencing	Amount of DNA in the Panel Member	"Mutation Detected" Rate (n=24)
10% FFPET Blend	9%	125 ng/25 µL	100%
		62.5 ng/25 µL	100%
		31.3 ng/25 µL	100%
5% FFPET Blend 1	5%	125 ng/25 µL	96%
		5.0 ng/25 µL	100%
		2.5 ng/25 µL	100%
		1.3 ng/25 µL	75%
		0.6 ng/25 µL	88%
		0.3 ng/25 µL	71%
5% FFPET Blend 2	5%	125 ng/25 µL	100%
		5.0 ng/25 µL	92%
		2.5 ng/25 µL	100%
		1.3ng/25 µL	96%
		0.6 ng/25 µL	58%
		0.3 ng/25 µL	50%
5% FFPET Blend 3	6%	125 ng/25 µL	100%
		5.0 ng/25 µL	100%
		2.5 ng/25 µL	100%
		1.3 ng/25 µL	100%
		0.6 ng/25 µL	96%
		0.3ng/25 µL	71%
2.5% FFPET Blend	3%	125 ng/25 µL	0%
		62.5 ng/25 µL	4%
		31.3 ng/25 µL	4%
0% (Wild-Type)	- - -	125 ng/25 µL	0%

This study demonstrates that the **cobas** BRAF Test can detect the BRAF V600E mutation at ≥ 5% mutation level using the standard input of 125 ng/25 µL. The ability of the Test to detect the mutation at lower DNA input levels demonstrates that the specimens can contain degraded DNA from the fixation process and still be detected. All test results obtained for the BRAF wild-type specimen were "Mutation Not Detected".

Analytical Sensitivity Using FFPET Specimens

To confirm the 5% mutation detection claim in patient specimens, forty-eight individual 5-µm sections from each of 3 BRAF V600E mutant FFPET specimens containing 6%, 12%, and 4% mutation levels were individually processed using 3 lots of **cobas**® DNA Sample Preparation Kit to isolate the DNA. To assess the impact of melanin on the assay, one specimen (6% mutation) had a high melanin concentration. Serial dilutions of the DNA from each section were prepared to produce a set of 6 panel members detailed in Table 5.

Table 5
Preparation of Dilution Panel Members from FFPET Specimens

FFPET Specimen	Specimen Information		Amount of DNA in Dilution Panel Members (ng/25 µL)
	Mean % V600E Mutation*	Pigmentation	
Specimen 1	6%	Highly Pigmented**	125, 15.6, 7.8, 3.9, 2.0, 1.0
Specimen 2	12%	NHP***	125, 7.8, 3.9, 2, 1, 0.5
Specimen 3	4%	NHP	125, 31.3, 15.6, 7.8, 3.9, 2.0

* *Mean percent mutation of the specimen determined by 454 sequencing*
** *Highly pigmented based on visual assessment, Melanin concentration = 0.17 µg /25 µL*
*** *NHP = Not Highly Pigmented based on visual assessment*

Sixteen (16) replicates of each panel member were run using each of 3 **cobas** BRAF Test kit lots (n=48/panel member). Sensitivity for each FFPET specimen was determined by the lowest amount of DNA that gave a BRAF V600E "Mutation Detected" rate of at least 95% (shaded rows). The results of the study are shown in Table 6.

Table 6
Sensitivity of the cobas BRAF Test using FFPET Specimens

FFPET Specimen	Percent Mutation by 454 Sequencing	Amount of DNA in the Panel Member	"Mutation Detected" Rate (n=48)
Specimen 1	6%	125 ng/25 µL	100%
		15.6 ng/25 µL	100%
		7.8 ng/25 µL	98%
		3.9 ng/25 µL	98%
		2.0 ng/25 µL	81%
		1.0 ng/25 µL	71%
Specimen 2	12%	125 ng/25 µL	100%
		7.8 ng/25 µL	100%
		3.9 ng/25 µL	100%
		2.0 ng/25 µL	98%
		1.0 ng/25 µL	98%
		0.5 ng/25 µL	94%
Specimen 3	4%	125 ng/25 µL	98%
		31.3 ng/25 µL	98%
		15.6 ng/25 µL	85%
		7.8 ng/25 µL	90%
		3.9 ng/25 µL	90%
		2.0 ng/25 µL	67%

The study demonstrated that the **cobas** BRAF Test can detect the BRAF V600E mutation in actual clinical FFPET specimens at ≥5% mutation level using the standard input of 125 ng/25 µL. The ability of the test to detect the mutation at lower DNA input levels demonstrates that the specimens can contain degraded DNA from the fixation process and still be detected. One highly pigmented specimen included in the study did not appear to affect the sensitivity of the test.

Analytical Sensitivity Using Cell Line Blend

DNA stocks from two melanoma cell lines [SK-MEL 28 (BRAF V600E mutant) and SK-MEL 2 (BRAF wild-type)] were blended to achieve a sample at 5% mutation, verified by 454 sequencing. Three separate dilution panels containing from 125 ng/25 µL to zero ng/25 µL DNA were prepared. Twenty (20) replicates of each panel member were tested, using each of 3 **cobas** BRAF Test kit lots (60 replicates total). Sensitivity was determined by the lowest amount of DNA that gave a BRAF V600E "Mutation Detected" rate of at least 95% (shaded row). The results of the study are shown in Table 7.

Table 7
Sensitivity of the cobas BRAF Test using Cell Line Blend

Cell Line Blend	Mean Percent Mutation by 454 Sequencing	Amount of DNA in the Panel Member	"Mutation Detected" Rate (n=60)
Cell Line Blend	5%	125.0 ng/25 µL	97%
		31.3 ng/25 µL	100%
		15.6ng/25 µL	95%
		7.8 ng/25 µL	98%
		3.9 ng/25 µL	95%
		2.0 ng/25 µL	82%
		1.0 ng/25 µL	78%
		0.5 ng/25 µL	77%

The **cobas** BRAF test gave a 95% "Mutation Detected" rate at 3.9 ng/25 µL, which represents 1:32 dilution of the recommended DNA input of 125 ng/25 µL. This would indicate that the test will detect the BRAF V600E mutation when ~97% of the DNA is degraded due to the fixation process, assuming that the cell line DNA contained 100% intact and amplifiable DNA.

Genomic Input Range:

The recommended DNA input for the **cobas** BRAF Test is 125 ng. Various genomic DNA input amounts may result from DNA quantitation errors and/or variation in the amount of degraded DNA. To evaluate the effects of various genomic DNA input amounts, genomic DNA was extracted from 11 melanoma FFPET specimens, selected for their mutation status and level of pigmentation, and serially diluted with sample input representing 250 ng, 125 ng, 62.5 ng, and 31.3 ng/ 25 µL. All 4 DNA levels were evaluated using 2 lots. The expected results were obtained for all genomic DNA input levels.

Minimal Tumor Content

Thirty-three (33) BRAF V600E mutant specimens, were tested to determine the minimum tumor proportion required for detecting the BRAF V600E mutation in specimens with tumor content ranging from 5% to 50%, without macro-dissection. One (1) section from each specimen was tested using the **cobas** BRAF Test.

The **cobas** BRAF Test correctly detected all BRAF V600E mutant specimens that had a minimum % mutant DNA above 5% and when the minimum tumor content was at least 15% as shown in Table 8. Specimens with less than 15% tumor content and less than 5% mutation level were reported as mutation-not-detected. An additional 24 wild type specimens with tumor content ranging from 5 to 45% were evaluated at the recommended DNA input concentration of 125 ng/25 µL, as well. All wild type specimens were correctly called. Macrodissection for specimens that contain < 50% tumor content by area is required.

Table 8

Results of Testing 33 BRAF V600E Specimens with Various Percent Tumor Content and Percent Mutation

Specimen Number	Tumor Content*	%Mutation	Test Result
1	5% / 5%	3%	Mutation Not Detected
2	5% / 5%	5%	Mutation Not Detected
3	5% / 5%	1%	Mutation Not Detected
4	10% / 10%	4%	Mutation Not Detected
5	10% / 10%	14%	Mutation Detected
6	15% / 10%	6%	Mutation Detected
7	15% / 15%	23%	Mutation Detected
8	15% / 15%	3%	Mutation Detected
9	15% / 15%	29%	Mutation Detected
10	15% / 15%	14%	Mutation Detected
11	15% / 15%	14%	Mutation Detected
12	15% / 20%	5%	Mutation Detected
13	20% / 20%	28%	Mutation Detected
14	20% / 20%	2%	Mutation Detected
15	25% / 20%	13%	Mutation Detected
16	25% / 25%	25%	Mutation Detected
17	30% / 25%	20%	Mutation Detected
18	30% / 30%	10%	Mutation Detected
19	30% / 35%	4%	Mutation Detected
20	30% / 35%	17%	Mutation Detected
21	35% / 30%	8%	Mutation Detected
22	35% / 35%	7%	Mutation Detected
23	35% / 35%	12%	Mutation Detected
24	35% / 35%	22%	Mutation Detected
25	35% / 40%	36%	Mutation Detected
26	40% / 35%	7%	Mutation Detected
27	40% / 35%	12%	Mutation Detected
28	40% / 40%	14%	Mutation Detected
29	40% / 40%	21%	Mutation Detected

Specimen Number	Tumor Content*	%Mutation	Test Result
30	40% / 40%	28%	Mutation Detected
31	40% / 45%	36%	Mutation Detected
32	45% / 45%	10%	Mutation Detected
33	50% / 40%	8%	Mutation Detected

** Tumor content of the specimen was assessed by examining the first and last of twelve adjacent 5-µm sections of each specimen by a pathologist. The tumor content of both the first and the last section is shown (for example, 95% / 95%).*

Cross-Reactivity

Cross-reactivity of the **cobas** BRAF Test was evaluated by testing the following specimen types;

- BRAF non-V600E mutant melanoma FFPET specimens at various mutation levels,
- Plasmids of BRAF non-V600E mutations,
- Plasmids of BRAF homologs,
- Skin-related microorganisms.

Cross-reactivity was also evaluated by determining whether the presence of BRAF homolog plasmids or skin-related microorganisms interfered with detection of the BRAF V600E mutation.

BRAF Non-V600E Melanoma FFPET Specimens

Fourteen (14) melanoma FFPET specimens with BRAF non-V600E mutations (V600D, V600E2, V600R, or V600K) were tested in triplicate with the **cobas** BRAF Test. For eight of the BRAF non-V600E specimens, all three replicates showed cross-reactivity with the **cobas** BRAF Test. These eight specimens were BRAF V600D mutant (18% mutation), BRAF V600E2 mutant (68% mutation), or BRAF V600K mutant (greater than 30% mutation). No cross-reactivity was observed for the BRAF V600R mutant (23% mutation) specimen as shown in Table 9.

Table 9
cobas BRAF Test Mutation Detected Rates Observed for BRAF Non-V600E Mutations in FFPET Specimens

Specimen Number	BRAF Mutation Status	Percent Mutation	Tumor Content*	Tumor Stage	Mutation Detected Rate (n=3)
1	V600D	18%	30% / 30%	IV	100%
2	V600E2	16%	75% / 75%	IV	0%
3		36%	75% / 80%	III	0%
4		68%	75% / 75%	IV	100%
5	V600R	23%	15% / 15%	IV	0%
6	V600K	17%	25% / 25%	III	0%
7		22%	35% / 40%	IV	0%
8		23%	40% / 40%	IV	0%
9		31%	60% / 60%	IV	100%
10		35%	75% / 75%	IV	100%
11		39%	80% / 80%	IV	100%
12		36%	95% / 95%	IIC	100%
13		62%	75% / 75%	IV	100%
14		69%	80% / 80%	IV	100%

** Tumor content of the specimen was assessed by examining the first and last of twelve adjacent 5-µm sections of each specimen by a pathologist. The tumor content of both the first and the last section is shown (for example, 95% / 95%).*

An eleven-member dilution panel with DNA concentrations ranging from 5.0 ng/µL down to 0.0049 ng/µL (corresponding to between 125 to 0.1 ng of DNA in the 25 µL input volume for the test) was prepared and each panel member tested in triplicate to determine the lowest amount of DNA that gave a 100% Mutation Detected Rate for the eight specimens found to cross react in the **cobas** BRAF Test. The lowest DNA input level before a loss in cross reactivity was observed ranged from 0.5 ng/25 µL for a BRAF V600K mutant specimen with 69% mutation to 15.6 ng/25 µL for a BRAF V600D mutant specimen with 18% mutation (Table 10).

Table 10
Lowest DNA Input to Detect Cross Reactivity of cobas BRAF Test

Specimen Number	BRAF Mutation Status	Percent Mutation	Lowest Amount of DNA Input Before Loss of Cross-Reactivity (n=3)
1	V600D	18%	15.6 ng/25 µL
2	V600E2	68%	7.8 ng/25 µL
3	V600K	31%	3.9 ng/25 µL
4		35%	3.9 ng/25 µL
5		39%	3.9 ng/25 µL
6		36%	2.0 ng/25 µL
7		62%	3.9 ng/25 µL
8		69%	0.5 ng/25 µL

BRAF Non-V600E Plasmids

Plasmid dilution panels with mutation levels ranging from 5% to 75% in a background of wild-type plasmid, were prepared for the following nine BRAF non-V600E mutations: D594G, G596R, K601E, L597Q, L597S, V600D, V600E2, V600K, and V600R. Three replicates of each member of the dilution panels prepared for each plasmid were tested using the **cobas** BRAF Test. Cross-reactivity was seen in all 3 replicates for BRAF V600D plasmid at ≥ 10% mutation, BRAF V600K plasmid at ≥ 35% mutation, and BRAF V600E2 plasmid at ≥ 65% mutation. No cross-reactivity was observed with plasmids from the six other BRAF mutations tested.

Plasmids of BRAF Homologs

Samples were prepared for three BRAF homolog plasmids (BRAF Pseudogene, ARAF, and RAF1), BRAF V600E mutant plasmid, and BRAF wild-type plasmid as outlined in Table 11. Three to six replicates of each panel member were tested using the **cobas** BRAF Test.

Table 11
BRAF Homolog Plasmid Samples

Panel		Composition by Volume	
Name	**Member**	**Component 1**	**Component 2**
BRAF Pseudogene	1	95% BRAF Pseudogene	5% BRAF V600E Mutant
	2	100% BRAF Pseudogene	- - -
ARAF	1	95% ARAF	5% BRAF V600E Mutant
	2	100% ARAF	- - -
RAF1	1	95% RAF1	5% BRAF V600E Mutant
	2	100% RAF1	- - -
Control	1	95% BRAF Wild-type	5% BRAF V600E Mutant
	2	100% BRAF Wild-type	- - -
	3	95% DNA Elution Buffer	5% BRAF V600E Mutant

None of the three BRAF homolog plasmids tested were detected by the **cobas** BRAF Test when tested alone, indicating that the BRAF homolog plasmids do not cross-react with the test.

The BRAF V600E Mutant plasmid at 5% in the presence of 95% of the BRAF homolog plasmids gave the expected “Mutation Detected” results in all cases, indicating that the homolog plasmids did not interfere with detection of the BRAF V600E mutation.

Skin-related Microorganisms

The following skin-related microorganisms were found to not cross react in the **cobas** BRAF Test when added to a wild-type melanoma FFPET specimen at 1 x 10^6 colony forming units (CFU) during the tissue lysis step :

1. *Staphylococcus epidermidis*
2. *Staphylococcus aureus*
3. *Corynebacterium xerosis*
4. *Corynebacterium jeikeium*
5. *Corynebacterium minutissimum*
6. *Corynebacterium ulcerans*

The tested microorganisms also did not interfere with detection of an FFPET specimen with 8% BRAF V600E mutation when 1 x 10^6 colony forming units (CFU) were added during the tissue lysis step.

Interference

Triglycerides ($\leq$ 74mM, 2x CLSI recommended high concentration[17]), hemoglobin ($\leq$ 2 mg/mL, 1x CLSI recommended high concentration[17]), and $\leq$ 95% necrotic tissue, did not to interfere with the **cobas** BRAF Test when the potential interfering substance was added to the lysis step during the specimen preparation procedure.

Melanin

The impact of high concentrations of endogenous melanin was evaluated using highly pigmented melanoma FFPET samples. A total of 41 unique FFPET melanoma tumor tissue specimens were selected based upon their level of pigmentation: 33 were highly pigmented, 3 were from African Americans and 5 were lightly pigmented for comparison. DNA was extracted from the tissue and melanin concentration was determined for each sample. A single replicate of the DNA stock from each of the two sections obtained from each of the 41 specimens was tested. Three specimens produced invalid results. One specimen produced a "Mutation Not Detected" result but this specimen was determined to be below the limit of detection. The 3 specimens with "Invalid" results were used to prepare the recommended concentration of DNA for the test as well as two-fold, four-fold, and eight-fold dilutions of the recommended DNA input of 125 ng/PCR. The resulting diluted DNA samples (containing a total of 125 ng, 61.5 ng, 31.25 ng, or 15.6 ng DNA in the 25 µL) were retested to determine if the corresponding reduction in melanin by dilution allowed valid results to be obtained. All three specimens when diluted 2-fold yielded the correct results.

Table 12
Summary of cobas BRAF Test Performance with Pigmented Melanoma FFPET Specimens

Specimen ID	Dilution	Melanin amount in Sample/PCR	Result
1	None (125 (ng)	0,15 µg	Invalid/Invalid
	Two-fold (62.5 ng)	0.08 µg	Mutation Detected/Mutation Detected
	Four-fold (31.3 ng)	0.04 µg	Mutation Detected/Mutation Detected
	Eight-fold (15.6 ng)	0.02 µg	Mutation Detected/Mutation Detected
2	None (125 (ng)	0.24 µg	Invalid/Invalid
	Two-fold (62.5 ng)	0.12 µg	Mutation Detected/Mutation Detected
	Four-fold (31.3 ng)	0.06 µg	Mutation Detected/Mutation Not Detected
	Eight-fold (15.6 ng)	0.03 µg	Mutation Not Detected/Invalid
3	None (125 (ng)	0.34 µg	Invalid/Invalid
	Two-fold (62.5 ng)	0.17 µg	Mutation Detected/Mutation Detected
	Four-fold (31.3 ng)	0.08 µg	Mutation Detected/Mutation Detected
	Eight-fold (15.6 ng)	0.04 µg	Mutation Detected/Mutation Detected

Results of testing the 17 wild-type specimens showed that all of the samples were correctly assigned a "Mutation Not Detected" result with the exception of 2 highly pigmented samples which yielded false positive results.

CLINICAL PERFORMANCE

Reproducibility

A study was performed to assess the reproducibility of the **cobas** BRAF Test across 3 external testing sites (2 operators per site), 3 reagent lots, and 5 non-consecutive testing days, with an 8-member panel of DNA samples derived from FFPET sections of malignant melanoma. This panel included both pigmented and non-pigmented samples and a range of percent tumor content and percent mutant alleles, including one sample at the 5% limit of detection (LOD). Of 94 runs, 92 (97.9%) were valid. Of 1442 samples tested, 2 samples (0.14%) gave invalid results. For all of the panel members except for the LOD samples, the correct call was made for 100% of valid tests, including samples panel members with 20% mutation, and two panel members determined to be highly pigmented. For the LOD panel member, the V600E mutation was detected in 90% (162/180) of samples. There were no false positives for any WT sample tested. In summary, the **cobas** BRAF Test was highly reproducible in both pigmented and non-pigmented samples, in samples with low tumor content and with low percent mutant alleles, and across testing sites, operators, reagent lots and testing days. Analytic specificity was 100%.

Correlation to Reference Method for Phase III Clinical Trial Specimens

The prevalence of the V600E mutation in the Phase III clinical trial was 46.5% based on results with the **cobas** BRAF Test. This is consistent with the prevalence of V600E in melanoma patients as reported in the literature.

To evaluate the performance of the **cobas** BRAF Test when compared to 2X bi-directional Sanger sequencing, 596 consecutive patients screened for the Phase III trial of vemurafenib were identified for whom clinical, demographic, and Sanger sequencing data were collected. Of these cases, 94 were ineligible because of missing inclusion criteria, 4 cases were without pathology review, 2 cases had invalid **cobas** BRAF Test results. Of the remaining eligible 496 cases, 47 specimens had invalid Sanger sequencing results, leaving 449 evaluable cases. The agreement analysis between the **cobas** BRAF Test results and Sanger sequencing results for the detection of the V600E mutation is shown in Table 13 below.

Table 13
Summary of cobas BRAF Test Results vs. Sanger Sequencing

	Sanger Sequencing (Reference Method)		
cobas BRAF Test (Test Method)	***BRAF V600E Mutation Detected[a]***	***BRAF V600E Mutation Not Detected[b]***	***Total***
Mutation Detected	216	35	251
Mutation Not Detected	6	192	198
Total	222	227	449
Positive Percent Agreement (95% CI)	100% x 216/222 = 97.3% (94.2%, 98.8%)		
Negative Percent Agreement (95% CI)	100% x 192/227 = 84.6% (79.3%, 88.7%)		
Overall Percent Agreement (95% CI)	100% x 408/449 = 90.9% (87.8%, 93.2%)		

a Mutation Detected indicates the presence of the predominant BRAF mutation type, V600E (1799 T>A), as identified by Sanger.

b Mutation Not Detected indicates the absence of the predominant BRAF mutation type, V600E, as identified by Sanger (ie, wild-type or no mutation, V600D, "V600E2", V600K, V600R and Other Mutations).

Note: Melanoma specimens with valid paired results from both the **cobas** BRAF Test and Sanger sequencing.

Note: CI = (score) confidence interval.

All 41 specimens which gave discordant **cobas** BRAF Test and Sanger sequencing results were subjected to 454 sequencing (quantitative massively parallel pyrosequencing) as a second reference method. Additionally, 33 **cobas** BRAF Test and Sanger sequencing-concordant specimens were tested with 454 sequencing. The secondary agreement analysis, after discordant resolution, is presented in Table 14.

Of the 6 discordant specimens which had a Mutation Not Detected result by the **cobas** BRAF Test and a V600E result by Sanger sequencing, 454 sequencing gave a wild-type result in 5/6 specimens (one specimen had an invalid 454 result).

Of the 8/35 discordant specimens which had a Mutation Detected result by the **cobas** BRAF Test and a WT result by Sanger sequencing, 454 sequencing detected a V600E mutation in 7/8 specimens (one specimen had an invalid 454 result).

Of the 27/35 discordant specimens which had a Mutation Detected result by the **cobas** BRAF Test and a non-V600E result by Sanger sequencing, 454 sequencing detected a V600K mutation in 24 specimens, a “V600E2” in one specimen and a V600E in one specimen. In 1 specimen, Sanger detected a V600D mutation and 454 sequencing gave a wild-type result.

The cross-reactivity of the **cobas** BRAF Test for V600K was 66% (25/38).

The agreement with 454 sequencing was 100% for the 33 **cobas** BRAF Test and Sanger sequencing-concordant V600E and WT specimens.

Table 14
cobas BRAF Test Results vs. Sanger Sequencing after Discordant Resolution by 454 Sequencing

cobas BRAF Test (Test Method)	***After Discordant Resolution by 454 Sequencing***		
	BRAF V600E Mutation Detected	***BRAF V600E Mutation Not Detected***	***Total***
Mutation Detected	224	27	251
Mutation Not Detected	1	197	198
Total	225	224	449
Positive Percent Agreement (95% CI)	100% x 224/225 = 99.6% (97.5%, 99.9%)		
Negative Percent Agreement (95% CI)	100% x 197/224 = 87.9% (83.0%, 91.6%)		
Overall Percent Agreement (95% CI)	100% x 421/449 = 93.8% (91.1%, 95.7%)		

Distribution of BRAF Codon 600 Mutations

The distribution of codon 600 mutations was determined in the 496 eligible cases based on a composite of Sanger and 454 sequencing results. Of these 496 cases, 182 cases were wild-type and 314 cases were mutant. The distribution of codon 600 mutations among 314 mutation positive cases is depicted in Table 15. V600K mutations were identified in 13.4% of all cases with codon 600 mutations.

Table 15
Distribution of BRAF Codon 600 Mutations of the Mutation-Positive Population as Determined by Sanger and/or 454 Sequencing

Amino Acid Sequence (Codon 600)	***Nucleotide Sequence (1798-1800)***	***N***	***% Distribution***
V600E	GAG	255	81.2
V600K	AAG	42	13.4
V600E2	GAA	13	4.1
V600R	AGG	3	1.0
V600D	GAC	1	0.3
Total codon 600 mutants		314	100
Total codon 600 wild type		182	---

ZELBORAF® (vemurafenib) Clinical Efficacy[9]

The **cobas** BRAF Test was used as a companion test for selecting patients for treatment with ZELBORAF®. The clinical safety and effectiveness of ZELBORAF® was demonstrated in the NO25026 study (BRIM3), an international, randomized, open-label, controlled, multicenter, Phase III study in previously untreated patients with unresectable stage IIIC or stage IV melanoma with a V600E BRAF mutation to evaluate the clinical efficacy of ZELBORAF® versus dacarbazine (standard of care therapy). FFPET samples from all melanoma patients being considered for treatment were tested with the **cobas** BRAF Test. Patients with a "Mutation Detected" test result were eligible for enrollment in the drug trial if they met other eligibility criteria. Patients with a "Mutation Not Detected" test result were ineligible for drug trial enrollment. The study was conducted at approximately 104 sites world-wide (22 centers in the US).

The trial enrolled 675 patients; 337 were allocated to receive vemurafenib and 338 to receive dacarbazine. The major efficacy outcome measures of the trial were overall survival (OS) and investigator-assessed progression-free survival (PFS). Other outcome measures included confirmed investigator-assessed best overall response rate.

Baseline characteristics were balanced between treatment groups. Most patients were male (56%) and Caucasian (99%), the median age was 54 years (24% were ≥ 65 years), all patients had ECOG performance status of 0 or 1, and the majority of patients had metastatic disease (95%).

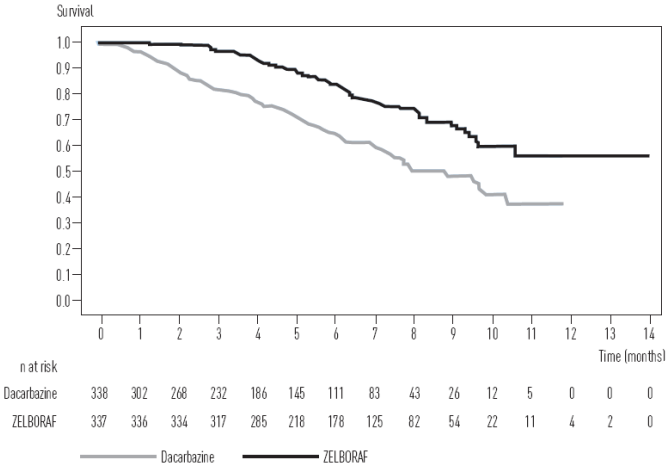
Efficacy results from the trial are shown below in Table 16 and Figure 2:

Table 16
Efficacy of Vemurafenib in Treatment-Naive Patients with BRAFV600E Mutation-Positive Melanoma[a]

	Vemurafenib (N=337)	**Dacarbazine (N=338)**	**p-value[d]**
Overall Survival			
Number of Deaths	78 (23%)	121 (36%)	-
Hazard Ratio (95% CI)[b]	0.44 (0.33, 0.59)		<0.0001
Median Survival (months) (95 % CI)[c]	NR[e] (9.6,NR)	7.9 (7.3, 9.6)	-
Median Follow-up (months) (range)	6.2 (0.4, 13.9)	4.5 (<0.1, 11.7)	
Progression-free survival Hazard Ratio (95% CI)[b]	0.26 (0.20, 0.33)		<0.0001
Median PFS (months)[c]	5.3 (4.9, 6.6)	1.6 (1.6, 1.7)	-

[a]As detected by the **cobas** BRAF Test
[b]Hazard ratio estimated using Cox model; a hazard ratio of < 1 favors vemurafenib
[c]Kaplan-Meier estimate
[d]Unstratified log-rank test
[e]Not Reached

Figure 2
Kaplan-Meier Curves of Overall Survival – Treatment Naive Patients



The confirmed, investigator-assessed best overall response rate was 48.4% (95% CI: 41.6%, 55.2%) in the ZELBORAF® arm compared to 5.5% (95% CI: 2.8%, 9.3%) in the dacarbazine arm.

COTELLIC® (cobimetinib) Clinical Efficacy[10, 11]

COTELLIC®, a MEK inhibitor, was tested in a clinical trial (coBRIM) in combination with ZELBORAF® compared to ZELBORAF® plus placebo. The **cobas** BRAF Test was used to determine eligibility for enrollment of patients in this clinical trial. The safety and efficacy of the combination of COTELLIC® and ZELBORAF® was established in a multicenter, randomized (1:1), double-blinded, placebo-controlled trial conducted in 495 patients with previously untreated, BRAF V600 mutation-positive, unresectable or metastatic, melanoma. The major efficacy outcome was investigator-assessed progression-free survival (PFS) per RECIST v1.1. Additional efficacy outcomes were investigator-assessed confirmed objective response rate (ORR), overall survival (OS), PFS as assessed by blinded independent central review, and duration of response (DOR).

Baseline characteristics were balanced between treatment groups. Most patients were male (58%) and Caucasian (93%), the median age was 55 years, 72% of patients had ECOG performance status of 0, and 60% of patients had stage M1c disease.

FFPET samples from all patients being considered for treatment were tested with the **cobas** BRAF Test. Patients with a "Mutation Detected" result were eligible for enrollment in the trial if they met other eligibility criteria. Patients with a "No Mutation Detected" result were ineligible for enrollment in the trial. The trial included patients with BRAF V600K mutations as detected by the **cobas** BRAF Test, demonstrating therapeutic product safety and efficacy in the detected portion of the patient population with tumors containing V600K.

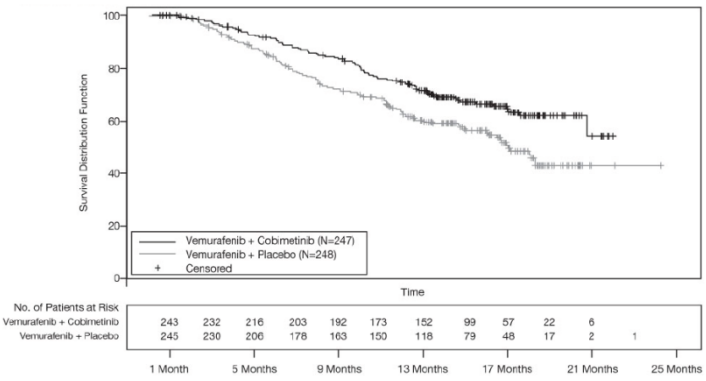
Efficacy results from the trial are shown below in Table 17 and Figure 3:

Table 17
Efficacy of Cobimetinib in Combination with Vemurafenib in Patients with BRAF Mutation-Positive Melanoma[a]

	Cobimetinib + Vemurafenib (N=247)	**Placebo + Vemurafenib (N=248)**	**p-value**
Progression-free survival (Investigator-assessed)			
Number of events (%)	143 (58%)	180 (73%)	
Progression	131	169	
Death	12	11	
Median PFS (months) (95% CI)	12.3 (9.5, 13.4)	7.2 (5.6, 7.5)	
Hazard Ratio (95% CI)[b]	0.56 (0.45, 0.70)		<0.001[d]
Overall Survival			
Number of Deaths (%)	79 (32%)	109 (44%)	
Median Survival (months) (95 % CI)[c]	Not estimable (20.7, Not estimable)	17.0 (15.0, Not estimable)	-
Hazard Ratio (95% CI)[b]	0.63 (0.47, 0.85)		0.0019[d,e]
Objective Response Rate			
Objective Response Rate (95 % CI)[c]	70% (64%, 75%)	50% (44%, 56%)	<0.001
Complete Response	16%	10%	
Partial Response	54%	40%	
Median Duration of Response, months (95 % CI)[c]	13.0 (11.1, 16.6)	9.2 (7.5, 12.8)	-

[a]As detected by the **cobas** BRAF Test
[b]Hazard ratio estimated using Cox model; a hazard ratio of < 1 favors cobimetinib + vemurafenib
[c]Kaplan-Meier estimate
[d]Stratified log-rank test
[e]Statistical significance depending on the comparison to the allocated alpha of 0.019 for this interim analysis

Figure 3
Kaplan-Meier Curves of Overall Survival



Available tumor samples from randomized patients were retrospectively analyzed using next generation sequencing (NGS) to further classify the BRAF mutations as V600E or V600K; test results were obtained on 81% of randomized patients (400/495). Among those patients for whom NGS was successfully performed, 56 out of 400 (14%) patients had tumors with BRAF V600K mutations, and the remaining patients had tumors with BRAF V600E mutations. The 56 tumors that were retrospectively found to have the BRAF V600K mutation had mutation frequencies between 5.1 and 36.6% in this analysis. A trend favoring the cobimetinib with vemurafenib arm was observed in exploratory subgroup analyses of PFS, OS, and ORR for the BRAF V600 mutation subtypes in the 81% of patients in this trial where BRAF V600 mutation type was determined.

II. PAPILLARY THYROID CARCINOMA (PTC)

NON-CLINICAL PERFORMANCE EVALUATION

For the nonclinical studies described below, tumor characteristics such as % tumor content were assessed by pathology review. Bi-directional Sanger sequencing was used to select the specimens for testing. The % mutation level was determined using 454 sequencing (quantitative massively-parallel pyrosequencing method).

Analytical Senstivity

Analytical Sensitivity- Limit of Detection (LoD)

The minimum amount of input DNA that produces correct results 95% of the time was assessed using dilution panels prepared from two types of specimens:

- Specimen blends prepared by mixing DNA stocks obtained from BRAF V600E mutant FFPET specimens and BRAF wild-type FFPET specimens to achieve specific mutation levels.
- Individual FFPET DNA stocks prepared from two BRAF V600E mutant 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 Using Specimen blends

BRAF V600E mutant FFPET specimen DNA stocks were blended with BRAF wild-type FFPET specimen DNA stocks to achieve one specimen at ~10%, one specimen at ~5%, and one specimen at ~2% mutation level. After blending, the mutation levels were verified by 454 sequencing. Each of the three specimen blends with V600E mutation were then diluted to produce the panel members detailed in Table 18.

Table 18
Preparation of Dilution Panel Members from Specimen Blends

Blend	Mean % Mutation*	Amount of DNA in Dilution Panel Members (ng/25 µL)**
10% Blend	10%	125, 41.7, 13.9, 4.6, 1.5, 0.5, 0.2, 0.1
5% Blend	5%	125, 41.7, 13.9, 4.6, 1.5, 0.5, 0.2, 0.1
2.5% Blend	2%	125, 41.7, 13.9, 4.6, 1.5, 0.5, 0.2, 0.1
0% (Wild-type only)	- - -	125

* *Mean percent mutation of the blend, tested by 454 sequencing*
** *Amount of genomic DNA contained in each panel member. 25 µL is the sample input volume for the test.*

Eight (8) replicates of each panel member were run using each of 3 **cobas** BRAF Test kit lots (n=24/panel member). Table 19 shows the sensitivity of each FFPET blend, determined by the lowest amount of DNA that gave a BRAF V600E "Mutation Detected" rate of at least 95% (shaded rows).

Table 19
Sensitivity of the cobas BRAF Test using FFPET Blends

FFPET Blend	Percent Mutation by 454 Sequencing	Amount of DNA in the Panel Member	"Mutation Detected" Rate (n=24)
10% FFPET Blend	10%	125 ng/25 µL	100%
		41.7 ng/25 µL	100%
		13.9 ng/25 µL	100%
		4.6 ng/25 µL	100%
		1.5 ng/25 µL	100%
		0.5 ng/25 µL	92%
		0.2 ng/25 µL	83%
		0.1 ng/25 µL	29%
5% FFPET Blend	5%	125 ng/25 µL	96%
		41.7 ng/25 µL	100%
		13.9 ng/25 µL	100%
		4.6 ng/25 µL	100%
		1.5 ng/25 µL	83%
		0.5 ng/25 µL	54%
		0.2 ng/25 µL	67%
		0.1 ng/25 µL	25%
2.5% FFPET Blend	2%	125 ng/25 µL	0%
		41.7 ng/25 µL	0%
		13.9 ng/25 µL	4%
		4.6 ng/25 µL	21%
		1.5 ng/25 µL	21%
		0.5 ng/25 µL	33%
		0.2 ng/25 µL	13%
		0.1 ng/25 µL	8%
0% (Wild-Type)	- - -	125 ng/25 µL	0%

This study demonstrates that the **cobas** BRAF Test can detect the BRAF V600E mutation at ≥5% mutation level using the standard input of 125 ng/25 µL. The ability of the Test to detect the mutation at lower DNA input levels demonstrates that the specimens can contain degraded DNA from the fixation process and still be detected. All test results obtained for the BRAF wild-type specimen were "Mutation Not Detected".

Analytical Sensitivity Using FFPET Specimens

To confirm the 5% mutation detection claim in patient specimens, twenty-four individual 5-µm sections from two BRAF V600E mutant FFPET specimens containing 6%, and 11% mutation levels were individually processed using 3 lots of **cobas**® DNA Sample Preparation Kit to isolate the DNA. Serial dilutions of the DNA from each section were prepared to produce a set of 8 panel members detailed in Table 20.

Table 20
Preparation of Dilution Panel Members from FFPET Specimens

	Mean % V600E Mutation*	Amount of DNA in Dilution Panel Members (ng/25 µL)
Specimen 1	6%	125, 41.7, 13.9, 4.6, 1.5, 0.5, 0.2, 0.1
Specimen 2	11%	125, 41.7, 13.9, 4.6, 1.5, 0.5, 0.2, 0.1

**Mean percent mutation of the specimen determined by 454 sequencing*

Eight (8) replicates of each panel member were run using each of 3 **cobas** BRAF Test kit lots (n=24/panel member). Sensitivity for each FFPET specimen was determined by the lowest amount of DNA that gave a BRAF V600E "Mutation Detected" rate of at least 95% (shaded rows). The results of the study are shown in Table 21.

Table 21
Sensitivity of the cobas BRAF Test using FFPET Specimens

FFPET Specimen	Percent Mutation by 454 Sequencing	Amount of DNA in the Panel Member	"Mutation Detected" Rate (n=48)
Specimen 1	6%	125 ng/25 µL	100%
		41.7 ng/25 µL	100%
		13.9 ng/25 µL	100%
		4.6 ng/25 µL	83%
		1.5 ng/25 µL	71%
		0.5 ng/25 µL	29%
		0.2 ng/25 µL	17%
		0.1 ng/25 µL	0%
Specimen 2	11%	125 ng/25 µL	100%
		41.7 ng/25 µL	100%
		13.9 ng/25 µL	100%
		4.6 ng/25 µL	71%
		1.5 ng/25 µL	46%
		0.5 ng/25 µL	17%
		0.2 ng/25 µL	13%
		0.1 ng/25 µL	8%

The study demonstrated that the **cobas** BRAF Test can detect the BRAF V600E mutation in actual clinical FFPET specimens at ≥ 5% mutation level using the standard input of 125 ng/25 µL. The ability of the test to detect the mutation at lower DNA input levels demonstrates that the specimens can contain degraded DNA from the fixation process and still be detected.

Repeatability

A study was performed to assess the repeatability of the **cobas** BRAF Test across two reagent lots, two operators, and four testing days, with five Papillary Thyroid Cancer FFPET specimens. These FFPET specimens included a range of percent tumor content (50 to 70%) and percent mutant alleles (16 to 22%), including two V600E mutant specimens at ~16-18% mutation (~3x LOD). The correct call was made for 100% of samples tested (80/80). There were no false positives for any WT sample tested. In summary, the **cobas** BRAF Test was highly repeatable in samples with low tumor content and with low percent mutant alleles, and across operators, reagent lots and testing days.

Correlation to Reference Method

To evaluate the performance of the **cobas** BRAF Test when compared to 2X bi-directional Sanger sequencing, 159 PTC FFPET specimens had Sanger sequencing data collected. The primary agreement analysis between the **cobas** BRAF Test results and Sanger sequencing results for the detection of the V600E mutation is shown in Table 22 for one of the two reagent lots tested. The second lot yielded similar results, except for one specimen that gave a Mutation Not Detected result. Resolution by 454 sequencing determined that the specimen contained 1.4% mutation and was below the 5% sensitivity claim for the **cobas** BRAF Test.

Table 22
Summary of cobas BRAF Test Results vs. Sanger Sequencing

	Sanger Sequencing (Reference Method)		
cobas BRAF Test (Test Method)	***BRAF V600E Mutation Detected[a]***	***BRAF V600E Mutation Not Detected[b]***	***Total***
Mutation Detected	88	13	101
Mutation Not Detected	1	57	58
Total	89	70	159
Positive Percent Agreement (95% CI)	100% x 88/89 = 98.9% (93.9%, 99.8%)		
Negative Percent Agreement (95% CI)	100% x 57/70 = 81.4% (70.8%, 88.8%)		
Overall Percent Agreement (95% CI)	100% x 145/159 = 91.2% (85.8%, 94.7%)		

[a] Mutation Detected indicates the presence of the predominant BRAF mutation type, V600E (1799 T>A), as identified by Sanger.

[b] Mutation Not Detected indicates the absence of the predominant BRAF mutation type, V600E, as identified by Sanger (i.e., wild-type or no mutation, and Other Mutations).

Note: CI = (score) confidence interval.

All specimens which gave discordant **cobas** BRAF Test and Sanger sequencing results were subjected to 454 sequencing (quantitative massively parallel pyrosequencing) as a second reference method. The secondary agreement analysis, after discordant resolution, is presented in Table 23.

One discordant specimen had a Mutation Not Detected result by the **cobas** BRAF Test and a V600E result by Sanger sequencing. 454 sequencing gave a wild-type result concordant with the **cobas** BRAF Test.

For twelve of the thirteen discordant specimens which had a mutation detected by the **cobas** BRAF Test and a wild-type by Sanger sequencing, 454 sequencing gave a V600E mutation (1.2% to 19% allele frequency), concordant with the **cobas** BRAF Test.

The one remaining discordant specimen which had a V600E Mutation Detected by the **cobas** BRAF Test was wild-type by Sanger sequencing and was also wild-type by 454 sequencing. An additional 454 sequencing investigation later confirmed the specimen to be a low percent V600E mutant.

Table 23
cobas BRAF Test Results vs. Sanger Sequencing after Discordant Resolution by 454 Sequencing

	Sanger Sequencing after Discordant Resolution by 454 Sequencing		
cobas BRAF Test (Test Method)	***BRAF V600E Mutation Detected***	***BRAF V600E Mutation Not Detected***	***Total***
Mutation Detected	101	1	102
Mutation Not Detected	0	57	57
Total	101	58	159
Positive Percent Agreement (95% CI)	100% x 101/101 = 100.0% (96.3%, 100.0%)		
Negative Percent Agreement (95% CI)	100% x 57/58 = 98.3% (90.9%, 99.7%)		
Overall Percent Agreement (95% CI)	100% x 158/159= 99.4% (96.5%, 99.9%)		

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 the following Table 24. Table 25 lists all result interpretation flags that are relevant for the user.

Table 24 Flag source

Flag code starts with	Flag source	Example
M*	Multiple or other reasons	M6
R	Result interpretation	R200
Z*	Analyzer	Z1

* Refer to the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System - User Assistance

Table 25 List of result interpretation flags

Flag Code	Severity	Description	Recommended Action
R200	Error	Mutant control invalid.	Repeat the run. Refer to the test-specific package insert. This flag code indicates that either: 1. An observed elbow value for the mutant control was below the established threshold (i.e. elbow too low). This may occur in the event of DNA contamination or an algorithm error due to atypical fluorescence pattern, or 2. An observed elbow value for the mutant control was above the established threshold (i.e. elbow too high). This may occur in the event of 1) incorrect preparation of working master mix, 2) pipetting error when adding working master mix into a reaction well of the microwell plate, or 3) pipetting error when adding mutant control into a reaction well of the microwell plate.
R201	Error	Wild-type control invalid.	Repeat the run. Refer to the test-specific package insert. This flag code indicates that either: 1. An observed elbow value for the wild-type control was below the established threshold (i.e. elbow too low). This may occur in the event of DNA contamination or an algorithm error due to atypical fluorescence pattern, or 2. An observed elbow value for the wild-type control was above the established threshold (i.e. elbow too high). This may occur in the event of 1) incorrect preparation of working master mix, 2) pipetting error when adding working master mix into a reaction well of the microwell plate, or 3) pipetting error when adding wild-type control into a reaction well of the microwell plate.
R202	Error	Mutant Ct not detected.	Repeat the sample. Refer to the test-specific package insert. This flag code indicates that a mutant elbow value was not observed for the sample. This may indicate the absence of the mutation in the sample or could indicate one or more of the following: 1. Low percentage of mutant sequences which are below the detection limit of the test. 2. Poor quality genomic DNA from the sample. 3. Inadequate sample processing. 4. Presence of PCR inhibitors in the sample. 5. Rare mutations within the regions of the genomic DNA covered by the primers and/or mutant probe. 6. Sample pipetting error or sample DNA may have not been added to the reaction well.

Flag Code	Severity	Description	Recommended Action
R203	Error	Wild-type Ct not detected.	Repeat the sample. Refer to the test-specific package insert. This flag code indicates that a wild-type elbow value was not observed for the sample. The absence of a wild-type elbow value is suggestive of one or more of the following: 1. Poor quality genomic DNA from the sample. 2. Inadequate sample processing. 3. The presence of PCR inhibitors in the sample. 4. Rare mutations within the regions of the genomic DNA covered by the primers and/or Wild-Type probe. 5. Sample DNA may have not been added to one or more wells.
R204	Error	Mutant Ct out of range.	Repeat the sample. Refer to the test-specific package insert. This flag code indicates that either: 1. An observed mutant elbow value for the sample was below the established threshold (i.e. elbow too low). This may occur if the PCR mixture is significantly overloaded with concentrated genomic DNA or due to an algorithm error due to atypical fluorescence pattern, or 2. An observed mutant elbow value for the sample was above the established threshold (i.e. elbow too high). This could indicate one or more of the following: • Low percentage of mutant sequences which are below the detection limit of the test. • Pipetting error in addition of sample DNA to the reaction well. • Poor quality genomic DNA from the sample. • Inadequate sample processing. • Presence of PCR inhibitors in the sample. • Rare mutations within the regions of the genomic DNA covered by the primers and/or mutant probe.
R205	Error	Wild-type Ct out of range.	Repeat the sample. Refer to the test-specific package insert. This flag code indicates that either: 1. An observed wild-type elbow value for the sample was below the established threshold (i.e. elbow too low). This may occur if the PCR mixture is significantly overloaded with concentrated genomic DNA or due to an algorithm error due to atypical fluorescence pattern, or 2. An observed wild-type elbow value for the sample was above the established threshold (i.e. elbow too high). This could indicate one or more of the following: • Pipetting error in addition of sample DNA to the reaction well. • Poor quality genomic DNA from the sample. • Inadequate sample processing. • Presence of PCR inhibitors in the sample. • Rare mutations within the regions of the genomic DNA covered by the primers and/or wild-type probe.

REFERENCES

1. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature. 2002; 417:949-54.
2. Bauer J, Büttner P, Murali R, et al. BRAF mutations in cutaneous melanoma are independently associated with age, anatomic site of the primary tumor, and the degree of solar elastosis at the primary tumor site. Pigment Cell Melanoma Res. 2011;24:345-51.
3. Curtin JA, Fridlyand J, Kageshita T, et al. Distinct sets of genetic alterations in melanoma. N Engl J Med. 2005; 353:2135-47
4. Kimura ET, Nikiforova MN, Zhu Z, et al. High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. Cancer Res. 2003; 63:1454–7.
5. Soares P, Trovisco V, Rocha AS, et al. BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. Oncogene. 2003; 22:4578–80.
6. Pollock PM, Harper UL, Hansen, KS, et al. High frequency of BRAF mutations in nevi. Nature Genet. 2003; 33:19-20.
7. COSMIC database (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic), Release v.57 (July 2012)
8. Flaherty KT, Puzanov I, Kim KB, et al. Inhibition of mutated, activated BRAF in metastatic melanoma. N Engl J Med. 2010; 363:809-19.
9. Chapman PB, Hauschild A, Robert C, et al. Improved survival with vemurafenib in melanoma with BRAF V600E mutation. N Engl J Med. 2011;364:2507-16.
10. Ascierto PA, McArthur GA, Dréno B, et al. Cobimetinib combined with vemurafenib in advanced BRAF(V600)-mutant melanoma (**coBRIM**): updated efficacy results from a randomised, double-blind, phase 3 trial. Lancet Oncol. 2016;17:1248-60.
11. COTELLIC (cobimetinib) U.S. package insert, Version 2.1, 2016.
12. Siroy AE, Boland GM, Milton DR, et al. Beyond BRAF(V600): clinical mutation panel testing by next-generation sequencing in advanced melanoma. J Invest Dermatol. 2015;135:508–15.
13. Longo MC, Berninger MS and Hartley JL. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions. Gene. 1990;93:125-8.
14. Chosewood LC and Wilson DE Biosafety and microbiological and biomedical laboratories. HHS Publication Fifth edition. (CDC) 21-1112. 2009.
15. Clinical and Laboratory Standards Institute (CLSI). Protection of laboratory workers from occupationally acquired infections. Approved Guideline-Fourth Edition. CLSI Document M29-A4: Wayne, PA;CLSI, 2014.
16. International Air Transport Association. Dangerous goods regulations, 60th Edition. 2019.
17. Clinical and Laboratory Standards Institute (CLSI). Interference testing in clinical chemistry. Approved Guideline-Second Edition. CLSI Document EP7-A2 Appendix D: Wayne, PA;CLSI, 2005

Document Revision Information	
Doc Rev. 10.0 08/2019	Updated **INTENDED USE** and **SUMMARY AND EXPLANATION OF TEST** sections. Added **ZELBORAF® (vemurafenib) Clinical Efficacy** and **COTELLIC® (cobimetinib) Clinical Efficacy** sections. Added slide stability information to the **SPECIMEN COLLECTION, TRANSPORT, AND STORAGE** section. Added melanin statement to the **PROCEDURAL LIMITATIONS** section. Added general language updates to clarify and harmonize with **cobas®** 4800 BRAF V600 Mutation Test US-IVD IFU. Please contact your local Roche Representative if you have any questions.
11/2019	Corrected text "FFFPET" to "FFPET" and the "greater than or equal to" symbol font on page 24 for proper PDF rendering. Updated the harmonized symbol page. Please contact your local Roche Representative if you have any questions.
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 beginning and in the Procedural Limitations section. Updated System and Operator Manuals throughout to "**cobas®** 4800 System – Operator's Manual or **cobas®** 4800 System – User Assistance". Made corrections to typos and updated for consistency and standardization of language throughout and for consistency to US IFU. Added Results flags. Updated International Air Transport Association reference. Updated the harmonized symbol page, trademarks and patents section, and distributors addresses. Added statement to clarify that product safety labeling primarily follows EU GHS guidance. Please contact your local Roche Representative if you have any questions.

Manufactured in the United States

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim, Germany
www.roche.com

Roche Diagnostics
9115 Hague Road
Indianapolis, IN 46250-0457 USA
(For Technical Assistance call the
Roche Response Center
toll-free: 1-800-526-1247)

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim, Germany

Trademarks and Patents

COBAS, COBAS Z, and AMPERASE are trademarks of Roche.

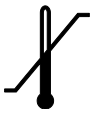
All other product names and trademarks are the property of their respective owners.

Carryover prevention technology in the AmpErase enzyme is covered by U.S. Patent 7,687,247 owned by Life Technologies and licensed to Roche Molecular Systems, Inc.

See http://www.roche-diagnostics.us/patents

06/2020 05952603001-12
Doc Rev. 11.0

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

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

US Customer Technical Support 1-800-526-1247