cobas® 4800 BRAF V600 Mutation Test

FOR *IN VITRO* DIAGNOSTIC USE.

cobas® DNA Sample Preparation Kit	DNA SP	24 Tests	P/N: 05985536190
cobas® 4800 BRAF V600 Mutation Test	BRAF	24 Tests	P/N: 05985579190

NOTICE: The purchase of this product allows the purchaser to use it for amplification and detection of nucleic acid sequences by polymerase chain reaction (PCR) and related processes for human in vitro diagnostics for the specific intended use stated in this Package Insert. No general patent or other license of any kind other than this specific right of use from purchase is granted hereby outside of the indications for use stated here.

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INTENDED USE

The cobas® 4800 BRAF V600 Mutation Test is an in vitro diagnostic device intended for the qualitative detection of the BRAF V600E mutation in DNA extracted from formalin-fixed, paraffin-embedded human melanoma tissue. The cobas® 4800 BRAF V600 Mutation Test is a real-time PCR test on the cobas 4800 system, and is intended to be used as an aid in selecting melanoma patients whose tumors carry the BRAF V600E mutation for treatment with 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] Mutations are also common in benign nevi,[4] suggesting that such mutations are a very early event. The discovery of such somatic mutations in the BRAF gene in melanoma 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 and other human tumors occur in codon 600.[5] 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 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[6,7].

PRINCIPLES OF THE PROCEDURE

The **cobas**® 4800 BRAF V600 Mutation 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**® 4800 BRAF V600 Mutation Test kit.

PCR Amplification and Detection

Target Selection

The **cobas**® 4800 BRAF V600 Mutation 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**® 4800 BRAF V600 Mutation Test is designed to detect the nucleotide (T1799A) 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 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®** 4800 BRAF V600 Mutation 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®** 4800 BRAF V600 Mutation Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP)[8]. 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 55°C, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon.

REAGENTS

	DNA SP	
cobas® DNA Sample Preparation Kit (P/N: 05985536190)		**24 Tests**
DNA TLB (DNA Tissue Lysis Buffer)		1 x 10 mL

Tris-HCl buffer
Potassium Chloride
0.04% EDTA
0.1% Triton X-100
0.09% Sodium azide

PK **1 x 100 mg**
(Proteinase K)

Proteinase K (lyophilized)

Xn  Proteinase K

Harmful

DNA PBB **1 x 10 mL**
(DNA Paraffin Binding Buffer)

Tris-HCl buffer
49.6% Guanidine hydrochloride
0.05% Urea
17.3% Triton X-100

Xn  49.6% (w/w) Guanidine HCl

Harmful

WB I **1 x 25 mL**
(DNA Wash Buffer I)

Tris-HCl buffer
64% Guanidine hydrochloride

Xn  64% (w/w) Guanidine HCl

Harmful

WB II **1 x 12.5 mL**
(DNA Wash Buffer II)

Tris-HCl buffer
Sodium chloride

DNA EB **1 x 6 mL**
(DNA Elution Buffer)

Tris-HCl buffer
0.09% Sodium azide

FT **1 x 25 pcs**
(Filter tubes with caps)

CT (Collection Tubes)	**3 x 25 pcs**
cobas® 4800 BRAF V600 Mutation Test	**24 Tests**
(P/N: 05985579190)	
RXNMIX (Reaction Mix)	**3 x 0.16 mL**
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	
MGAC (Magnesium acetate)	**3 x 0.15 mL**
Magnesium Acetate 0.09% Sodium azide	
BRAF OM (BRAF Oligo Mix)	**3 x 0.13 mL**
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	
BRAF MUT (BRAF Mutant Control)	**2 x 0.13 mL**
Tris-HCl buffer EDTA 0.09% Sodium azide < 0.001% plasmid DNA (microbial) containing BRAF mutant sequence < 0.001% plasmid DNA (microbial) containing BRAF wild-type sequence	
BRAF WT (BRAF Wild-Type Control)	**2 x 0.13 mL**
Tris-HCl buffer EDTA 0.09% Sodium azide < 0.001% plasmid DNA (microbial) containing BRAF wild-type sequence	

DNA SD **2 x 1 mL**
(DNA Specimen Diluent)

Tris-HCl buffer
0.09% Sodium azide

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. Material Safety Data Sheets (MSDS) 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.

L. **DNA PBB, WB I** and **WB II** contain guanidine hydrochloride. If liquid containing this buffer is spilled, clean with suitable laboratory detergent and water. If a spill occurs with potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 0.5% sodium hypochlorite*. If spills occur on the **cobas z** 480 analyzer, follow the instructions in the **cobas**® 4800 system Operator's Manual.

***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.

M. Specimens should be handled as infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories*[9] and in the CLSI Document M29-A3[10].

N. **DNA TLB** and **DNA PBB** contain Triton X-100, an irritant to mucous membranes. Avoid contact with eyes, skin and mucous membranes.

O. **DNA TLB, DNA PBB, DNA EB, 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.

P. Xylene is a hazardous chemical and should be used in a chemical hood. Discard into chemical waste in accordance with local, state, and federal regulations.

Q. 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.

R. All disposable items are single use. Do not reuse.

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

T. 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.

U. 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.

V. The use of sterile disposable pipets and DNase-free pipet tips is recommended.

STORAGE AND HANDLING REQUIREMENTS

A. Do not freeze reagents.

B. Store **DNA TLB**, **DNA PBB**, **WB I**, **WB II**, **DNA EB**, **FT** and **CT** at 15-30°C. Once opened, these reagents are stable for up to 8 uses over 90 days or until the expiration date, whichever comes first.

C. Store **PK** at 15-30°C. After addition of sterile, nuclease-free water to **PK**, store unused reconstituted **PK** in 450 µL aliquots at -20°C. Once reconstituted, **PK** must be used within 90 days or until the expiration date, whichever comes first.

D. After addition of absolute ethanol, store **WB I** and **WB II** at 15-30°C. These Working Solutions are stable for up to 8 uses over 90 days or until the expiration date, whichever comes first.

E. 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.

F. **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.

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

H. Processed specimens are stable for up to 24 hours at 15-30°C, up to 14 days at 2-8°C or frozen at -20°C for up to 60 days. The processed specimens (extracted DNA) are also stable after undergoing up to 4 freeze thaws.

I. 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

A. **cobas**® DNA Sample Preparation Kit 24 Tests **DNA SP**
(P/N: 05985536190)

DNA TLB
(DNA Tissue Lysis Buffer)

PK
(Proteinase K)

DNA PBB
(DNA Paraffin Binding Buffer)

WB I
(DNA Wash Buffer I)

WB II
(DNA Wash Buffer II)

DNA EB
(DNA Elution Buffer)

FT
(Filter tubes with caps)

CT
(Collection Tubes)

B. **cobas**® 4800 BRAF V600 Mutation Test 24 Tests **BRAF**
(P/N: 05985579190)

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

- Xylene (ACS, ≥98.5% xylenes)
- Absolute Ethanol (for Molecular Biology)
- Isopropanol (ACS, ≥995%)
- Sterile Nuclease-free Water (for Molecular Biology)
- Sterile disposable, serological pipettes: 5 and 25 mL
- **cobas**® 4800 System Microwell Plate (AD-Plate) and Sealing Film (Roche P/N 05232724001)
- Adjustable Pipettors*: (capacity 10 µL. 20 µL, 200 µL and 1000 µL) with aerosol barrier or positive displacement DNase-free tips
- Pipette Aid (Drummond P/N: 4-000-100 or equivalent)
- Bench top microcentrifuge capable of 20,000 x g
- Two (2) Dry Heat Blocks capable of heating microcentrifuge tubes to 56°C and 90°C**
- 1.5 mL Safe-Lock microcentrifuge tubes, sterile, RNase/DNase free, PCR grade
- NanoDrop UV-Vis Spectrophotometer (Thermo Scientific ND-1000 or ND-2000)**
- Vortex mixer**
- Microcentrifuge tube racks
- Disposable gloves, powderless
- Calibrated Thermometers for Dry Heat Block**
- Waterbath** capable of maintaining 37°C
- Single edge blade or similar

** 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
- BRAF Analysis Package Software version 1.0
- Barcode Reader (Roche P/N 05339910001)
- Printer HP P2055d (Roche P/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**® 4800 BRAF V600 Mutation Test.

B. Specimen Transport

FFPET specimens can be transported at 15-30°C. Transportation of FFPET specimens must comply with country, federal, state and local regulations for the transport of etiologic agents[11].

C. Specimen Storage

Stability of FFPET specimens stored at 15-30°C for up to 9 months after the date of collection has been confirmed. 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 FFPET sections of 5 µm thickness containing at least 50% tumor content are to be used in the cobas® 4800 BRAF V600 Mutation Test. Any specimen containing less than 50% tumor content should be macro-dissected prior to deparaffinization.

NOTE: Refer to the cobas® 4800 system Operator's Manual for detailed operating instructions for the cobas® z 480 analyzer.

NOTE: Dry heat blocks, capable of heating microcentrifuge tubes, should be turned on and set at 56°C and 90°C.

Run Size

The **cobas**® 4800 BRAF V600 Mutation 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 an insufficient volume of reagents to run a total of 24 specimens plus controls with the kit. The **cobas**® 4800 BRAF V600 Mutation Test contains reagents sufficient for 8 runs of 3 specimens plus controls. One replicate of the **cobas**® 4800 BRAF V600 Mutation Test Mutant Control **[BRAF MU]** and one replicate of the **cobas**® 4800 BRAF V600 Mutation Test Wild-type Control **[BRAF WT]** are required to perform each run (see *"Quality Control"* section).

Workflow

NOTE: cobas® 4800 BRAF V600 Mutation 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.

The **cobas**® 4800 BRAF V600 Mutation Test consists of manual sample preparation using the **cobas**® **DNA Sample Preparation Kit** followed by amplification/detection on the **cobas**® **z** 480 analyzer using the **cobas**® 4800 BRAF V600 Mutation Test kit. Run size can be from one specimen plus controls to 24 specimens plus controls.

Reagent Preparation

1. Reconstitute Proteinase K (**PK**) by adding 4.5 mL of sterile (PCR grade) water to the vial using a sterile, disposable 5-mL serological pipette. Mix by inverting the vial 5 to 10 times. Dispense 450-µL aliquots of reconstituted **PK** into 1.5 mL Safe-Lock microcentrifuge tubes and store at -20°C. If the Proteinase K has already been reconstituted and frozen, thaw a sufficient number of aliquots to process the number of specimens to be run prior to deparaffinization (70µL of reconstituted **PK** is required for each specimen).

2. All solutions stored at 15-30°C should be clear. If precipitate is present in any reagent, warm the solution in a 37°C water bath until the precipitate dissolves. Do not use until all precipitate has been dissolved.

3. Prepare working DNA Wash Buffer I (**WB I**) by adding 15 mL of absolute ethanol to the bottle of **WB I**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB I** at 15°C to 30°C.

4. Prepare working DNA Wash Buffer II (**WB II**) by adding 50 mL of absolute ethanol to the bottle of **WB II**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB II** at 15°C to 30°C.

Deparaffinization of FFPET Sections Mounted on Slides

*Note: **Xylene is a hazardous chemical. All steps for deparaffinization should be performed under a chemical hood. See Warnings and Precautions***

A. Add a slide with mounted 5 µm FFPET section to a container with sufficient xylene to cover tissue, and soak for 5 minutes.

B. Transfer slide to container with sufficient absolute ethanol to cover tissue and soak for 5 minutes.

C. Remove slide and allow section to air dry completely (5 to 10 minutes).

D. Perform macrodissection if specimen contains <50% tumor content

E. Label one 1.5-mL Safe-Lock microcentrifuge tube for each specimen with the specimen identification information.

F. Add 180 µL **DNA TLB** into the labeled 1.5-mL Safe-Lock microcentrifuge tube.

G. Add 70 µL of reconstituted **PK** to the 1.5-mL Safe-Lock microcentrifuge tube containing **DNA TLB**

H. Scrape the tissue off the slide and immerse into the **DNA TLB** and **PK** mixture

I. Continue to Step A of the **DNA Isolation** procedure.

Deparaffinization of FFPET Sections not Mounted on Slides

*Note: **Xylene is a hazardous chemical. All steps for deparaffinization should be performed under a chemical hood. See Warnings and Precautions***

A. Macrodissection for specimens that contains <50% tumor content is required. Place one 5-µm FFPET section into a 1.5-mL Safe-Lock microcentrifuge tube labeled with the specimen identification information for each specimen.

B. Add 500 µL Xylene to a Safe-Lock microcentrifuge tube containing the FFPET section.

C. Mix well by vortexing for 10 seconds.

D. Let the tube stand for 5 minutes at 15°C-30°C.

E. Add 500 µL absolute ethanol and mix by vortexing for 10 seconds.

F. Let the tube stand for 5 minutes at 15°C-30°C.

G. Centrifuge at 16,000 x g to 20,000 x g for 2 minutes and remove supernatant without disturbing the pellet. Discard the supernatant into chemical waste.

H. Add 1 mL absolute ethanol and vortex for 10 seconds.

I. Centrifuge at 16,000 x g to 20,000 x g for 2 minutes and remove the supernatant without disturbing the pellet. Discard the supernatant into chemical waste.

*NOTE: **If the pellet is floating in the remaining supernatant, spin again for 1 minute at 16,000 x g to 20,000 x g. Remove any remaining supernatant.***

J. Dry the tissue pellet for 10 minutes at 56°C in a heating block with tubes open.

433 ***NOTE: Make sure the ethanol is completely evaporated and pellet is dry before proceeding to the next step.***

434 ***NOTE: If needed, dry pellets can be stored up to 24 hours at 2 - 8°C.***

435 K. Resuspend tissue pellet in 180 µL of DNA Tissue Lysis Buffer (**DNA TLB**).

436 L. Add 70 µL of reconstituted **PK**.

437 M. Continue to Step A of the **DNA Isolation** procedure.

438 DNA Isolation

439 A. Vortex tube with specimen/**DNA TLB/PK** mixture for 30 seconds.

440 ***NOTE: The tissue must be fully immersed in the DNA TLB/PK mixture.***

441 B. Place tube in 56°C dry heat block and incubate for 60 minutes.

442 C. Vortex the tube for 10 seconds.

443 ***NOTE: The tissue must be fully immersed in the DNA TLB/PK mixture.***

444 D. Place tube in 90°C dry heat block and incubate for 60 minutes.

445 ***NOTE: During the incubation, prepare the required number of filter tubes (FT) with hinged caps by placing onto***
446 ***collection tubes (CT) and label each FT/CT unit with proper identification on the cap of each FT.***

447 ***NOTE: Each specimen will need 1 FT, 3 CT and one elution tube (1.5 mL microcentrifuge tube).***

448 ***NOTE: During the incubation, label the required number of elution tubes (1.5 mL microcentrifuge tubes) with proper***
449 ***specimen identification information.***

450 E. Allow the tube to cool to 15°C-30°C. After cooling, pulse centrifuge to collect any excess liquid from the cap.

451 F. Add 200 µL **DNA PBB** and mix by pipetting up and down 3 times.

452 G. Incubate tube at 15°C-30°C for 10 minutes.

453 H. Add 100 µL isopropanol and mix lysate by pipetting up and down 3 times.

454 I. Transfer all of the lysate into the appropriately labeled **FT/CT** unit.

455 J. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.

456 K. Place **FT** onto a new **CT**. Discard the flow-through from the old **CT** into chemical waste and properly dispose of the old **CT**.

457 L. Add 500 µL working **WB I** to the **FT**.

458 ***NOTE: Preparation of working WB I is described in the Reagent Preparation section.***

459 M. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.

460 N. Discard the flow-through in each **CT** into chemical waste. Place **FT** back into the same **CT**.

461 O. Add 500 µL working **WB II** to the **FT**.

462 ***NOTE: Preparation of working WB II is described in the Reagent Preparation section.***

463 P. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.

464 Q. Place **FT** onto a new **CT**. Discard the flow-through from the old **CT** into chemical waste and properly dispose of the old **CT**.

465 R. Centrifuge **FT/CT** unit at 16,000 to 20,000 x g for 1 minute to dry the filter membrane.

466 S. Place the **FT** tube into an elution tube (1.5 mL microcentrifuge tube) pre-labeled with specimen identification information. Discard
467 the flow-through from the old **CT** into chemical waste and properly dispose of the old **CT**.

468 T. Add 100 µL **DNA EB** to the center of the **FT** membrane without touching the **FT** membrane.

469 U. Incubate **FT** with elution tube at 15°C-30°C for 5 minutes.

V. Centrifuge **FT** with elution tube at 8,000 x g for 1 minute to collect eluate into the elution tube (pre-labeled 1.5-mL microcentrifuge tube). Properly dispose of the **FT**. The eluate is the **DNA stock**.

W. Close caps on elution tubes. Continue with Step A in the DNA Quantitation section.

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

DNA Quantitation

A. Mix each DNA stock by vortexing for 5 seconds before quantitation.

B. Quantify DNA by a NanoDrop UV-Vis Spectrophotometer (ND-1000 or ND-2000) according to the manufacturer's protocol. Use **DNA EB** as the blank for the instrument. An average of 2 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/ul, the two measurements should be within ± 2.0 ng/µL.

C. DNA stock concentration must be ≥ 5 ng/µL to perform the **cobas**® 4800 BRAF V600 Mutation Test.

NOTE: Each DNA stock must have a minimum concentration of 5 ng/µL to perform the cobas® 4800 BRAF V600 Mutation Test. If the concentration of a DNA stock is < 5 ng/µL, the DNA Isolation procedure should be repeated for that specimen using two 5 µm FFPET sections. Continue with Step A of "Deparaffinization of FFPET Sections Mounted on Slides" or Step A of "Deparaffinization of FFPET Sections Not Mounted on Slides" combining the tissue from both slides/sections into one tube. Continue with the DNA Isolation procedure. If the DNA stock is still < 5 ng/µL, another FFPET specimen may need to be requested from the referring clinical site.

NOTE: Store undiluted DNA stock at 2°C - 8°C for up to 2 weeks or at -20°C for up to 60 days

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 Instrument Operator's Manual for detailed instruction for the **cobas** z 480 set-up.

Test Order Set-Up

Refer to the **cobas** 4800 system Operator's Manual Software Version 2.0 for **cobas**® BRAF V600 Mutation Test (**cobas**® BRAF Operator's Manual) for detailed instructions on the BRAF workflow steps.

Dilution Calculation of Specimen DNA Stock

Only one amplification/detection is run per specimen, using 25 uL 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 uL 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 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 Safe-Lock 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 properly 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 as MMX 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 P/N 05232724001)

I. Carefully add 25 uL of working MMX to each reaction well of the microwell plate (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 uL of **BRAF MUT** Control to well **A01** of the microwell plate (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 uL of **BRAF WT** Control to well **B01** of the microwell plate (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 uL of diluted specimen DNA to the appropriate well containing working MMX, starting from position C01 on the microwell plate (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 microwell plate (AD-plate) within 1 hour after the preparation of the working Master Mix (MMX)

Figure 1

Sample Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	BRAF MUT	Sample 7	Sample 15	Sample 23								
B	BRAF WT	Sample 8	Sample 16	Sample 24								
C	Sample 1	Sample 9	Sample 17									
D	Sample 2	Sample 10	Sample 18									
E	Sample 3	Sample 11	Sample 19									
F	Sample 4	Sample 12	Sample 20									
G	Sample 5	Sample 13	Sample 21									
H	Sample 6	Sample 14	Sample 22									

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

E. Cover the microwell plate (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 microwell plate (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 Software Version 2.0 for **cobas**® 4800 BRAF V600 Mutation Test 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® 4800 BRAF V600 Mutation Test Result	Interpretation

Mutation Detected	V600E Mutation Detected in the BRAF codon 600 site in exon 15
Mutation Not Detected*	V600E 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 result does not preclude the presence of a mutation in the BRAF codon 600 site since results depend on percent mutant sequences, adequate specimen integrity, absence of inhibitors, and sufficient DNA to be detected.*

All of the patients in the phase III study NO25026 (BRIM3) sponsored by Hoffmann-La Roche were selected using the cobas® 4800 BRAF V600 Mutation Test. Enrollment in the study was limited to patients whose melanoma tissue tested positive by the test. Therefore it is not known whether patients who test negative by the cobas® 4800 BRAF V600 Mutation Test will benefit from vemurafenib (Zelboraf ™) treatment.

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 start with the "Deparaffinization of FFPET Sections Mounted on Slides" or "Deparaffinization of FFPET Sections not Mounted on Slides" procedure, 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 Deparaffinization and DNA Isolation 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**® 4800 BRAF V600 Mutation 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. In order for a mutant control to be called valid, the Ct value needs to be between 23.5 to 30.5 in the mutant channel, and between 23.5 to 29.0 in the wild-type channel. In order for a wild-type control to be called valid, the Ct value needs to be >43 in the mutant channel and between 31.5 to 38.5 in the wild-type channel. 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 microwell plate (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**® 4800 BRAF V600 Mutation Test has been validated for use only with melanoma FFPET specimens.
2. The **cobas**® 4800 BRAF V600 Mutation Test has been validated using only the **cobas**® DNA Sample Preparation Kit (Roche P/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.
5. The addition of AmpErase enzyme into the **cobas**® 4800 BRAF V600 Master 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 v2.0.
7. Only the **cobas**® 4800 System v2.0 has been validated for use with this product. No other PCR system has been validated with this product.
8. Though rare, mutations and variants within the regions of the BRAF gene covered by the primers or probes used in the **cobas**® 4800 BRAF V600 Mutation Test may result in failure to amplify the BRAF V600 allele or detect the presence of mutation in codon 600.
9. The presence of PCR inhibitors may cause false negative or invalid results.
10. 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".**
11. The **cobas**® 4800 BRAF V600 Mutation Test shows limited cross-reactivity with non-V600E mutant specimens (V600K, V600D, and V600E2). Refer to the Non-clinical Performance Evaluation section for more details.
12. FFPET specimens containing degraded DNA may affect the ability of the test to detect the V600E mutation.
13. The cobas 4800 BRAF mutation test is a qualitative test. The test is not for quantitative measurements of mutation.

NON-CLINICAL PERFORMANCE EVALUATION

For the nonclinical studies described below, % tumor was assessed by pathology review and melanin content was assessed by pathology review. Bi-directional Sanger sequencing was used to select the specimens for testing. The % mutation was determined using a parallel sequencing method.

Analytical Sensitivity – Limit of Blank (LoB)

To assess performance of the cobas 4800 BRAF V600 Mutation Test in the absence of template and to ensure that a blank sample, or a sample with an excess of 100% wild-type DNA, does not generate an analytical signal that might indicate a low concentration of mutation, samples with no template and 100% BRAF wild-type DNA were evaluated. No detectable Ct results were identified in the mutant channel in the presence of 100% BRAF wild-type DNA or in either channel following a no template sample. Additionally, no detectable Ct was identified in the wild-type channel when testing a 100% BRAF V600E sample.

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.

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. Each of the five specimen blends with V600E mutation (but not the wild-type specimen) was then diluted to produce panel members at a range of DNA concentrations (125 ng to 0.3 ng in a 25 uL volume).

Eight (8) replicates of each panel member were run using each of 3 **cobas**® 4800 BRAF V600 Mutation Test kit lots (n=24/panel member). This study demonstrated that in FFPET specimen blends, the **cobas**® 4800 BRAF V600 Mutation Test can detect the BRAF V600E mutation at ≥5% mutation level using the standard input of 125 ng/25 µL. The ~10% blend correctly called all replicates at a 31 ng/25 µL dilution. The 3% FFPET blend at 125 ng/25 µL was not detected.

Analytical Sensitivity Using FFPET Specimens

To confirm the 5% claim in patient specimens, forty-eight individual 5 µm sections from each of 3 BRAF V600E mutant FFPET specimens containing 5%, 6%, and 12%, 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 at a range of DNA concentrations (125 ng to 0.5 ng in a 25 µL volume).

Sixteen (16) replicates of each panel member were run using each of 3 **cobas**® 4800 BRAF V600 Mutation Test kit lots (n=48/panel member).

The study demonstrated that the **cobas**® 4800 BRAF V600 Mutation 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.

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. Three separate dilution panels containing from 125 ng/25 µL to 0.5 ng/25 µL DNA were prepared. Twenty (20) replicates of each panel member were tested, using each of 3 **cobas**® 4800 BRAF V600 Mutation 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%. The results of the study are shown in Table 3 below.

Table 3:
Sensitivity of the cobas® 4800 BRAF V600 Mutation Test using Cell Line Blend

Cell Line Blend	Mean Percent Mutation	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 Test gave a 95% "Mutation Detected" rate at 3.9 ng/25µL, which represents a 1:32 dilution of the recommended DNA input of 125 ng/25µL.

Genomic Input Range:

The recommended DNA input for the cobas® 4800 BRAF V600 Mutation 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**® 4800 BRAF V600 Mutation Test.

The **cobas**® 4800 BRAF V600 Mutation 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 4. Specimens whose tumor content was less than 15% tumor content and less than 5% mutation 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 in 25 µL as well. All wild-type specimens were correctly called. Macrodissection for specimens that contain <50% tumor content is required.

Table 4:
Results of Testing 33 BRAF V600E FFPET Specimens with Various Percent Tumor Content

V600E Specimen Number	Tumor Content*	Test Result
1	5% / 5%	Mutation Not Detected
2	5% / 5%	Mutation Not Detected
3	5% / 5%	Mutation Not Detected
4	10% / 10%	Mutation Not Detected
5	10% / 10%	Mutation Detected
6	15% / 10%	Mutation Detected
7	15% / 15%	Mutation Detected
8	15% / 15%	Mutation Detected
9	15% / 15%	Mutation Detected

10	15% / 15%	Mutation Detected
11	15% / 15%	Mutation Detected
12	15% / 20%	Mutation Detected
13	20% / 20%	Mutation Detected
14	20% / 20%	Mutation Detected
15	25% / 20%	Mutation Detected
16	25% / 25%	Mutation Detected
17	30% / 25%	Mutation Detected
18	30% / 30%	Mutation Detected
19	30% / 35%	Mutation Detected
20	30% / 35%	Mutation Detected
21	35% / 30%	Mutation Detected
22	35% / 35%	Mutation Detected
23	35% / 35%	Mutation Detected
24	35% / 35%	Mutation Detected
25	35% / 40%	Mutation Detected
26	40% / 35%	Mutation Detected
27	40% / 35%	Mutation Detected
28	40% / 40%	Mutation Detected
29	40% / 40%	Mutation Detected
30	40% / 40%	Mutation Detected
31	40% / 45%	Mutation Detected
32	45% / 45%	Mutation Detected
33	50% / 40%	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**® 4800 BRAF V600 Mutation 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**® 4800 BRAF V600 Mutation Test. For eight of the BRAF non-V600E specimens, all three replicates showed cross-reactivity with the **cobas**® 4800 BRAF V600 Mutation Test. These eight specimens were: BRAF V600D mutant (18% mutation), BRAF V600E2 mutant (68.0% 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 5.

Table 5:
cobas® BRAF V600 Mutation Test Mutation Detected Rates Observed for BRAF Non-V600E Mutations in FFPET Specimens*

Specimen Number	BRAF Mutation Status	Percent Mutation	Tumor Content[a]	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		40%	80% / 80%	IV	100%
12		36%	95% / 95%	IIC	100%
13		62%	75% / 75%	IV	100%
14		69%	80% / 80%	IV	100%

[a]*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%).*

*The cobas 4800 BRAF V600 Mutation Test does not reliably detect non-V600E mutations

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**® 4800 BRAF V600 Mutation 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 6. Three to six replicates of each panel member were tested using the **cobas**® 4800 BRAF V600 Mutation Test.

Table 6:
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**® 4800 BRAF V600 Mutation 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" result 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**® 4800 BRAF V600 Mutation 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 epidermis*
2. *Staphylococcus aureus*
3. *Corynbacterium xerosis*
4. *Corynebacterium jeikeium*
5. *Corynbacterium minutissimum*
6. *Corynbacterium 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 were added during the tissue lysis step.

Interference

Triglycerides (≤74mM, 2x CLSI recommended high concentration[12]), and hemoglobin (≤2 mg/mL, 1x CLSI recommended high concentration[12]), did not interfere with the **cobas**® 4800 BRAF V600 Mutation Test when the potential interfering substance was added to the lysis step during the specimen preparation procedure.

Necrotic tissue

The ability of the cobas® 4800 BRAF V600 Mutation Test to perform correctly when samples have high necrotic tissue content was tested using 27 V600E or WT melanoma FFPET specimens that contained from 10% to 95% necrotic tissue. Samples were selected based on their BRAF V600E mutation status, necrotic tissue content, and percent mutation. Of the 27 specimens selected, 11 were BRAF V600E mutant, and 16 were BRAF wild-type. The percent mutation ranged from 4% to 65%. The tumor content and percentage of necrotic tissue in each specimen was reviewed by a pathologist. Samples were run in duplicate using 1 lot of reagents. For the 11 BRAF V600E specimens, mutation detected results were obtained for all samples except one whose % mutation was below the threshold of detection (4%). The specimen with 95% necrotic tissue whose % mutation was 10% was correctly called mutation detected. Mutation not detected results were obtained for all wild-type specimens.

Table 7:
Summary of cobas 4800 BRAF V600 Mutation Test Performance with High Necrotic Melanoma FFPET Specimens

Specimen	% Necrosis	Test Results (n = 2)
V600E	15%	Mutation Detected/Mutation Detected
	15%	Mutation Detected/Mutation Detected
	20%	Mutation Detected/Mutation Detected
	25%	Mutation Detected/Mutation Detected
	25%	Mutation Detected/Mutation Detected
	30%	Mutation Detected/Mutation Detected
	30%	Mutation not Detected/Mutation not Detected
	55%	Mutation Detected/Mutation Detected
	55%	Mutation Detected/Mutation Detected
	60%	Mutation Detected/Mutation Detected
	95%	Mutation Detected/Mutation Detected

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.

797 *Table 8:*
798 *Summary of cobas 4800 BRAF V600 Mutation 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

799 Results of testing the 17 V600 wild-type specimens showed that all of the samples were correctly assigned a "mutation not detected"
800 result with the exception of 2 highly pigmented samples which yielded false positive results.

801 Reproducibility

802 An external study was performed to assess the reproducibility of the **cobas** 4800 BRAF V600 Mutation Test across 3 external
803 testing sites (2 operators per site), 3 reagent lots, and 5 non-consecutive testing days, with an 8-member panel of DNA
804 samples derived from FFPE sections of malignant melanoma. This panel included both pigmented and non-pigmented
805 samples and a range of percent tumor content and percent mutant alleles, including one sample at 4% (near the 5% limit of
806 detection (LOD)). Of 94 runs, 92 (97.9%) were valid. Of 1442 samples tested, 2 samples (0.14%) gave invalid results. For all
807 of the panel members except for the LOD samples, the correct call was made for 100% of valid tests, including samples panel
808 members with 20% mutation, and two panel members determined to be highly pigmented. For the LOD panel member, the
809 V600E mutation was detected in 90% (162/180) of samples. There were no false positives for any WT sample tested. Results
810 by overall agreement are presented below.

Table 9:
Overall Agreement Estimates by Panel Member in the cobas 4800 BRAF V600 Mutation Test Reproducibility Study

Panel Member	Number of Valid Tests	Agreement (%)	95% CI[a] for Agreement (%)
Wild-type-75% Tumor	180	100.0 (180/180)	(98.0, 100.0)
Wild-type-75% Tumor Pigmented	180	100.0 (180/180)	(98.0, 100.0)
V600E-5% Mutation	180	90.0 (162/180)	(84.7, 94.0)
V600E-20% Mutation	180	100.0 (180/180)	(98.0, 100.0)
V600E Mutant-50% Tumor	180	100.0 (180/180)	(98.0, 100.0)
V600E Mutant-50% Tumor Pigmented	180	100.0 (180/180)	(98.0, 100.0)
V600E Mutant-75% Tumor	180	100.0 (180/180)	(98.0, 100.0)
V600E Mutant-90% Tumor	180	100.0 (180/180)	(98.0, 100.0)

Note: Results are included as agreement when valid test of Mutant Type panel member has a result of "mutation detected" or when a valid test of a Wild-type panel member has a result of "mutation not detected".
[a] 95% CI=95% exact binomial confidence interval.

Across all components of variance (lot, site, operator, day, within-run), the CV (%) for the mutant and wild-type Cts were calculated and was <3% and <1.8%, respectively, for all panel members. The CV(%) for the external controls was ≤ 2.0%.

Repeatability

To evaluate the repeatability of the Test, a separate internal study was conducted. Duplicate results were obtained on five different melanoma FFPET specimens tested on four different days by two operators, using two different reagent lots and two cobas 4800 instruments. Four BRAF V600E-mutant specimens, two with tumor content less than 35%, two with mutation percentages less than 12% (~2X of the analytical sensitivity of the Test of 5% mutation detection), and one Wild-type specimen were selected for this study. On each of the four days, each of two operators tested four sections from each of the 5 FFPET melanoma specimens, two sections were tested per lot of DNA Specimen Preparation Kit and cobas 4800 BRAF V600 Mutation Test kit. A total of 32 replicates were evaluated per sample. Repeatability was considered acceptable if at least 95% of the calls corresponded to sequencing results for the specimens. A single replicate of one BRAF V600E mutant specimen (10% mutation) and a single replicate of another BRAF V600E mutant specimen (10% mutation) had results of "Mutation Not Detected." All other replicates for those specimens and all replicates for the remaining three specimens matched the expected results, corresponding to a correct call accuracy of 98.75% across the entire study.

Table 10:
Repeatability of the cobas 4800 BRAF V600 Mutation Test

Panel Member (% mutation)	% Tumor Content[a]	Tumor Stage	Total no. replicates tested	Total no incorrect calls	% accuracy
V600E (10%)	60% / 55%	IV	32	1	96.9
V600E (12%)	40% / 35%	IIIB	32	0	100
V600E (10%)	30% / 30%	IV	32	1	96.9
V600E (17%)	30% / 35%	II	32	0	100
WT	60% / 60%	IV	32	0	100

[a] 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%).

Correlation to Reference Method for Phase III Clinical Samples:

To evaluate the performance of the cobas® 4800 BRAF V600 Mutation test when compared to 2X bi-directional sequencing, 596 consecutive patients screened for the Phase III trial 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 test results and 47 specimens had invalid Sanger sequencing results leaving 449 evaluable cases. The agreement analysis between the cobas test results and Sanger sequencing results for the detection of the V600E mutation is shown in Table 21 below. The Positive Percent Agreement (PPA) with Sanger sequencing was 97.3% (216/222), and the Negative Percent Agreement (NPA) was 84.6% (192/227), with an overall Percent Agreement of 90.9%. There were a total of 35 mutations detected by the cobas test which were not identified as V600E mutations by Sanger sequencing. Eight (8) of these were wild-type, 25 were V600K and 2 were other codon 600 mutations by Sanger sequencing. Additionally, 6 specimens were identified as Mutation Not Detected by the cobas test but were identified as V600E by Sanger sequencing. The cross-reactivity of the cobas test for V600K was 66% (25/38).

Table 11:
*BRAF V600 Mutation Test vs. Sanger Sequencing for Specimens from Phase III Study**

Cobas 4800 BRAF Mutation Test Result	Sanger Sequencing (Reference Method)							
	BRAF V600E Mutation Detected	BRAF V600E Mutation Not Detected						Total
	V600E	V600K	V600E2	V600R	V600D**	V600 Other***	Wild-type	
Mutation Detected	216	25	1	0	1	0	8	251
Mutation Not Detected	6	13	12	2	0	1	164	198
Total	222	38	13	2	1	1	172	449

Positive Percent Agreement (95% CI)	216/222 X 100% = 97.3% (94.2%, 98.8%)
Negative Percent Agreement (95% CI)	192/227 X 100% = 84.6% (79.3%, 88.7%)
Overall Percent Agreement	408/449 X 100% = 90.9% (87.8%, 93.2%)

*Sanger sequencing has a limit of detection of approximately 20% mutant alleles in FFPET specimens[13,14,15,16]. Therefore, Sanger sequencing cannot be adequately used to confirm mutation status at lower percentages of mutant alleles. Consistent with this, in an exploratory analysis, some samples that were negative by Sanger sequencing and positive by cobas tested positive by a third method. The cobas test has a validated limit of detection in FFPET of 5% mutant alleles.

**V600D variant (GTG>GAC)
***Mutation not specified in the protocol (ACA insertion immediately before Codon 600)

The estimated prevalence of the V600E mutation in the Phase III clinical trial was 50 to 55% based on results with the cobas test. This is consistent with the prevalence of V600E in melanoma patients as reported in literature [3].

All of the patients in the phase III study NO25026 (BRIM3) sponsored by Hoffmann-La Roche were selected using the cobas® 4800 BRAF V600 Mutation Test. Enrollment in the study was limited to patients whose melanoma tissue tested positive by the test. Therefore it is not known whether patients who test negative by the cobas® 4800 BRAF V600 Mutation Test will benefit from vemurafenib (Zelboraf ™) treatment.

Vemurafenib Clinical Efficacy

The cobas® 4800 BRAF V600 Mutation Test was used as a companion test for selecting patients for treatment with vemurafenib (Zelboraf™). The clinical safety and effectiveness of vemurafenib (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 V600E BRAF mutation to evaluate the efficacy of vemurafenib (RO5185426) versus dacarbazine. Formalin-fixed paraffin-embedded tissue (FFPET) samples from all melanoma patients being considered for treatment were tested with the cobas® 4800 BRAF V600 Mutation Test. Patients with a test positive result for the mutation were eligible for enrollment in the drug trials if they met other eligibility criteria. Patients with a negative test result were ineligible for drug trial enrollment. The study was conducted at approximately 104 sites (22 centers in the US).

The trial enrolled 675 patients; 337 were allocated to receive ZELBORAF 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%).

Table 12:
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]	Not Reached (9.6, Not Reached)	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)	-

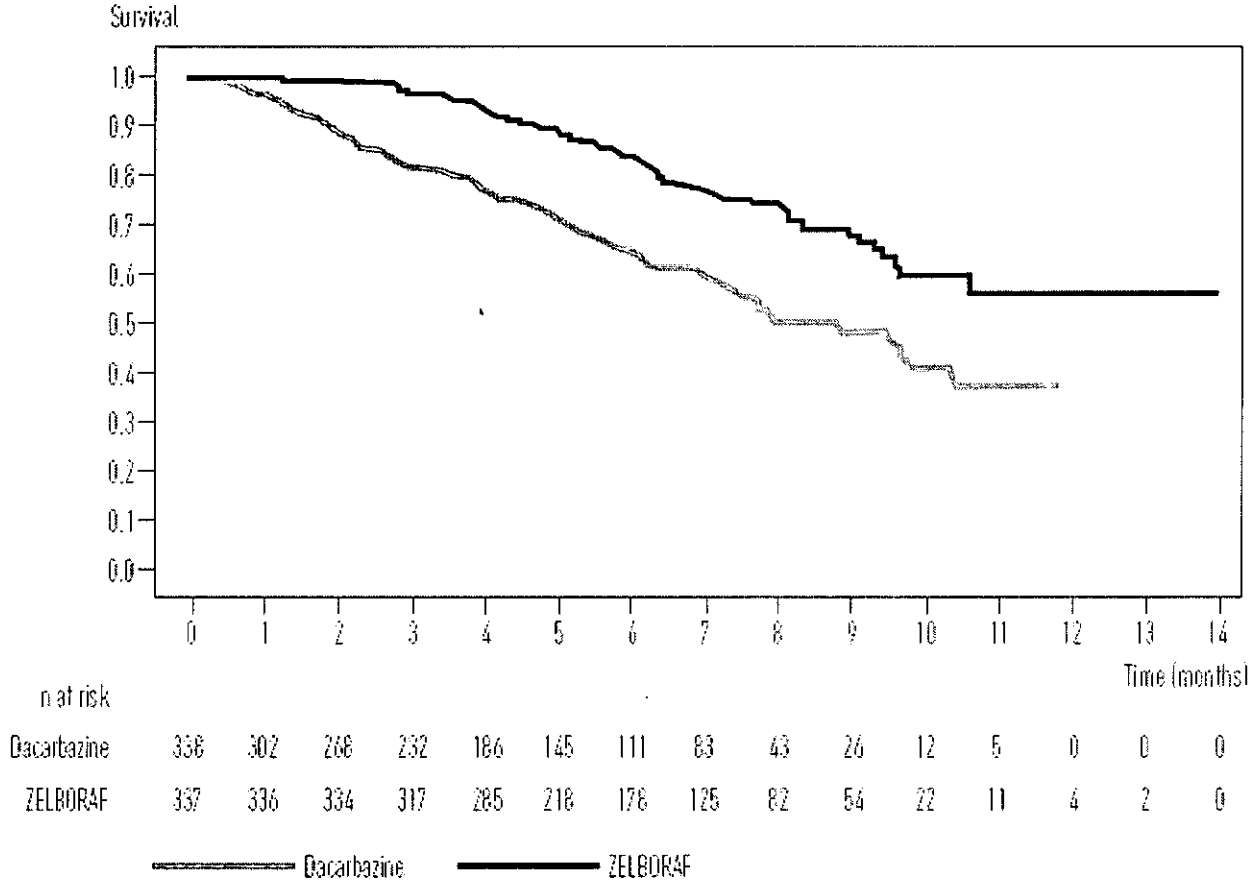
[a] As detected by the cobas® 4800 BRAF V600 Mutation Test

[b] Hazard ratio estimated using Cox model; a hazard ratio of < 1 favors vemurafenib

[c] Kaplan-Meier estimate

[d] Unstratified log-rank test

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.

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