

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

cobas [®] DNA Sample Preparation Kit	DNA SP	24 Tests	P/N: 05985536190
cobas [®] PIK3CA Mutation Test	PIK3CA	24 Tests	P/N: 06523013190

PRINCIPLES OF THE PROCEDURE

The **cobas[®]** PIK3CA Mutation Test is a research use only (RUO) real-time PCR test for the qualitative detection and identification of mutations in exons 1, 4, 7, 9, and 20 of the phosphoinositide-3-kinase, catalytic, alpha (PIK3CA) gene in DNA derived from formalin-fixed paraffin-embedded (FFPET) tissue. Samples are processed using the **cobas[®]** DNA Sample Preparation Kit for manual sample preparation and the **cobas z 480** analyzer for automated amplification and detection.

The **cobas[®]** PIK3CA Mutation Test is based on two major processes: (1) manual sample preparation to obtain genomic DNA from FFPET; and (2) PCR amplification and detection of target DNA using complementary primer pairs and oligonucleotide probes labeled with fluorescent dyes. The test is designed to detect R88Q in exon 1, N345K in exon 4, C420R in exon 7, E542K, E545X (E545A, E545D*, E545G, and E545K), Q546X (Q546E, Q546K, Q546L, and Q546R) in exon 9, and M1043I[†], H1047X (H1047L, H1047R, and H1047Y), and G1049R in exon 20 when the percent mutation is 5% or greater. Mutation detection is achieved through PCR analysis with the **cobas z 480** analyzer. A mutant control and a negative control are included in each run to confirm the validity of the run.

Sample Preparation

FFPET specimens are processed and genomic DNA isolated using the **cobas[®]** DNA Sample Preparation Kit, a generic manual sample preparation based on nucleic acid binding to glass fibers. A deparaffinized 5 µm section of an FFPET sample is lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released genomic DNA from DNases. Subsequently, isopropanol is added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the genomic DNA is bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and then eluted with an aqueous solution. The amount of genomic DNA is spectrophotometrically determined and adjusted to a fixed concentration to be added to the amplification/detection mixture. The target DNA is then amplified and detected on the **cobas z 480** analyzer using the amplification and detection reagents provided in the **cobas[®]** PIK3CA Mutation Test kit.

PCR Amplification

Target Selection

The **cobas[®]** PIK3CA Mutation Test kit uses a pool of primers that define specific base-pair sequences that range from 85 to 155 base pairs long in PIK3CA exons 1, 4, 7, 9, and 20. An additional primer pair targets a conserved 167 base pair region in exon 3 of the PIK3CA gene to provide a full process control for sample adequacy, extraction and amplification. Amplification occurs only in the regions of the PIK3CA gene between the primers; the entire PIK3CA gene is not amplified.

Target Amplification

A derivative of *Thermus* species Z05-AS1 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-AS1 DNA polymerase, in the presence of divalent metal ion and excess dNTP, extends each annealed primer, thus synthesizing a second DNA strand. This completes the first cycle of PCR, yielding a double-stranded DNA copy which includes the targeted base-pair regions of the PIK3CA gene. This process is repeated for a number of cycles, with each cycle effectively doubling the amount of amplicon DNA.

* For the E545D amino acid change, only the nucleotide change c.1635G>T mutation is detected by the test.

† For the M1043I amino acid change, only the nucleotide change c.3129G>T mutation is detected by the test.

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Automated Real-time Mutation Detection

The **cobas**[®] PIK3CA 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-AS1 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. Four different reporter dyes are used to detect the mutations targeted by the test. Amplification of the targeted PIK3CA sequences are detected independently across three reactions by measuring fluorescence at the four characteristic wavelengths in dedicated optical channels.

Selective Amplification

Selective amplification of target nucleic acid from the sample is achieved in the **cobas**[®] PIK3CA Mutation Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP)¹. The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing thymidine. Deoxyuridine is not present in naturally occurring DNA but is always present in amplicon due to the use of dUTP in addition to deoxythymidine triphosphate as one of the nucleotide triphosphates in the Master Mix reagents; 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 Master Mix reagents, 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 (Materials Provided)

cobas[®] DNA Sample Preparation Kit
(P/N: 05985536190)

DNA SP

24 Tests

DNA TLB

(DNA Tissue Lysis Buffer)

1 x 10 mL

PK

(Proteinase K)

Proteinase K (lyophilized)

1 x 100 mg

Xn Proteinase K



Harmful

DNA PBB

(DNA Paraffin Binding Buffer)

1 x 10 mL

Xn 49.6% (w/w) Guanidine HCl



Harmful

WB I

(DNA Wash Buffer I)

1 x 25 mL

Xn 64% (w/w) Guanidine HCl



Harmful

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WB II (DNA Wash Buffer II)		1 x 12.5 mL
DNA EB (DNA Elution Buffer)		1 x 6 mL
FT (Filter tubes with caps)		1 x 25 pcs
CT (Collection Tubes)		3 x 25 pcs
cobas® PIK3CA Mutation Test (P/N: 06523013190)	PIK3CA	24 Tests
PIK3CA MMX-1 (PIK3CA Master Mix 1; Cap with White Button)		2 x 0.48 mL
PIK3CA MMX-2 (PIK3CA Master Mix 2; Cap with Gold Button)		2 x 0.48 mL
PIK3CA MMX-3 (PIK3CA Master Mix 3; Cap with Teal Button)		2 x 0.48 mL
MGAC (Magnesium acetate; Cap with Yellow Button)		6 x 0.2 mL
PIK3CA MC (PIK3CA Mutant Control; Cap with Red Button)		6 x 0.1 mL
DNA SD (DNA Specimen diluent)		2 x 3.5 mL

WARNINGS AND PRECAUTIONS

- A. **For Research Use Only. Not for use in diagnostic procedures.**
- B. This test is for use with FFPET samples.
- 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 or if kits were stored improperly.
- H. Do not pool reagents from different kits or lots.
- I. Gloves must be worn and must be changed between handling samples and reagents to prevent contamination.
- J. To avoid contamination of the working Master Mix (working MMX, i.e. after addition of **MGAC** to the MMX) with DNA samples, amplification and detection should be performed in an area separated from DNA Isolation. The amplification and detection work area should be thoroughly cleaned before working MMX preparation. For proper cleaning, all surfaces including racks and pipettors should be thoroughly wiped with 0.5% sodium hypochlorite solution followed by wiping with a 70% ethanol solution.

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- K. **DNA PBB** and **WB I** 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 z 480** analyzer Instrument 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.**

- L. Samples should be handled as infectious using safe laboratory procedures such as those outlined in *Biosafety in Microbiological and Biomedical Laboratories*² and in the CLSI Document M29-A3³.
- M. **DNA TLB** and **DNA PBB** contain Triton X-100, an irritant to mucous membranes. Avoid contact with eyes, skin, and mucous membranes.
- N. **DNA TLB, DNA EB, MGAC, PIK3CA MMX-1, PIK3CA MMX-2, PIK3CA MMX-3, PIK3CA MC, 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.
- O. Xylene is a hazardous chemical and should be used in a chemical hood. Discard chemical waste in accordance with local, state, and federal regulations.
- P. 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.
- Q. All disposable items are for one time use. Do not reuse.
- R. Do not use disposable items beyond their expiration date.
- S. Do not use sodium hypochlorite solution (bleach) for cleaning the **cobas z 480** analyzer. Clean the **cobas z 480** analyzer according to procedures described in the **cobas z 480** analyzer Instrument Manual.
- T. Samples and/or reagents should not be transferred from one microwell plate to another microwell plate to reduce the risk of contamination.
- U. For additional warnings, precautions and procedures to reduce the risk of contamination for the **cobas z 480** analyzer, consult the **cobas z 480** analyzer Instrument Manual.
- V. The use of sterile disposable pipettes and DNase-free pipettor tips is recommended.

STORAGE AND HANDLING REQUIREMENTS

- A. With the exception of the reconstituted **PK** reagent, do not freeze reagents.
- B. Store **DNA TLB, DNA PBB, WB I, WB II, DNA EB, PK, FT, and CT** at 15°C to 30°C. Once opened, **DNA TLB, DNA PBB, WB I, WB II, DNA EB, and PK** are stable for up to 8 uses over 90 days or until the expiration date, whichever comes first.
- 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°C to 30°C. These working solutions are stable for 90 days or until the expiration date, whichever comes first.
- E. Store **MGAC, PIK3CA MMX-1, PIK3CA MMX-2, PIK3CA MMX-3, PIK3CA MC, and DNA SD** at 2°C to 8°C. Once opened, these reagents are stable for 4 uses within 90 days or until the expiration date, whichever comes first.
- F. **PIK3CA MMX-1, PIK3CA MMX-2, PIK3CA MMX-3**, and working MMX (prepared by the addition of **MGAC** to **PIK3CA MMX-1** or **PIK3CA MMX-2** or **PIK3CA MMX-3**) should be protected from prolonged exposure to light.

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- G. Store working MMX at room temperature in the dark. The prepared samples and controls must be added within 1 hour of preparation of the working MMX.
- H. Processed specimens (extracted DNA) are stable for up to 24 hours at 15°C to 30°C, up to 14 days at 2°C to 8°C, up to 60 days at -15°C to -25°C and after undergoing 3 freeze thaws when stored at -15°C to -25°C. Extracted DNA should be amplified within the recommended storage periods or before the expiration date of the **cobas**[®] DNA Sample Preparation Kit used to extract the DNA, whichever comes first.
- I. Amplification must be started within 1 hour from the time that the processed samples and controls are added to the working MMX (prepared by the addition of **MGAC** to **PIK3CA MMX-1** or **PIK3CA MMX-2** or **PIK3CA MMX-3**).

MATERIALS REQUIRED BUT NOT PROVIDED

- Xylene (Sigma-Aldrich, Cat.# 247642 or Thermo Fisher Scientific, Cat.# X5-4 or equivalent)
- Absolute ethanol (Sigma-Aldrich, Cat.# E7023 or Thermo Fisher Scientific, Cat.# BP2818-500 or equivalent)
- Isopropanol (Sigma-Aldrich, Cat.# 190764 or Thermo Fisher Scientific, Cat.# A451-1 or equivalent)
- Sterile water, nuclease-free PCR Grade Water (Life Technologies, Cat.# AM9937 or Thermo Fisher Scientific, Cat.# SH-30538.01 or equivalent)
- Sterile disposable, serological pipettes: 5 and 25 mL
- **cobas**[®] 4800 System Microwell Plate (AD-Plate) and Sealing Foil (Roche P/N 05232724001)
- **cobas**[®] 4800 Sealing Foil Applicator (Roche P/N 04900383001)
- Adjustable Pipettors* (capacity 10 µL, 20 µL, 200 µL, and 1000 µL) with aerosol barrier or positive displacement DNase-free tips
- Pipette aid (Drummond P/N: 4-000-100 or equivalent)
- Bench top microcentrifuge capable of 8,000 x g and 16,000 to 20,000 x g (Eppendorf 5417C or equivalent)**
- 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 (Eppendorf, Cat# 022363212)
- Nanodrop UV-Vis Spectrophotometer (Thermo Fisher Scientific ND-1000 or ND-2000)**
- Vortex mixer**
- Microcentrifuge tube racks
- Disposable gloves, powder-free
- Calibrated thermometers for dry heat block**
- Waterbath** capable of maintaining 37°C
- Single edged 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 sample degradation and cross-contamination.

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

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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
- PIK3CA Analysis Package Software version 1.0 or higher
- Barcode Reader ext USB
- Printer

SAMPLE COLLECTION, TRANSPORT, AND STORAGE

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

A. Sample Collection

FFPET samples may be used with the **cobas**[®] PIK3CA Mutation Test.

B. Sample Transport

FFPET samples can be transported at 15°C to 30°C. Transportation of FFPET samples must comply with country, federal, state, and local regulations for the transport of etiologic agents.⁴

C. Sample Storage

FFPET samples may be stored at 15°C to 30°C for up to 12 months after the date of tissue collection.

5 µm sections may be stored at 15°C to 30°C for up to 60 days.

INSTRUCTIONS FOR USE

NOTE: *Only FFPET sections of 5 µm thickness containing at least 10% tumor content by area are to be used in the **cobas**[®] PIK3CA Mutation Test. Any sample containing less than 10% tumor content by area should be macro-dissected after deparaffinization.*

NOTE: *Refer to the **cobas z** 480 analyzer Instrument 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

A single run can include from 1 to 30 samples (plus controls) per 96 well Microwell plate. When running more than 24 samples, multiple **cobas**[®] PIK3CA Mutation Test kits of the same lot will be required.

The **cobas**[®] PIK3CA Mutation Test contains sufficient reagents for 8 runs of 3 samples (plus controls) for a maximum of 24 samples per kit.

Workflow

The **cobas**[®] PIK3CA 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**[®] PIK3CA Mutation Test kit.

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Reagent Preparation

- A. Reconstitute Proteinase K (**PK**) by adding 4.5 mL of sterile, nuclease-free (PCR grade) water to the vial using a sterile, disposable 5-mL serological pipette. Mix by inverting the vial 5 to 10 times. Aliquot 450 μ L 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 sufficient number of aliquots to process the number of samples to be run prior to deparaffinization (70 μ L of reconstituted **PK** is required for each sample).
- B. All solutions stored at $15\text{--}30^{\circ}\text{C}$ 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.
- C. 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. Make a note on the bottle that ethanol has been added and the date. Store working **WB I** at 15°C to 30°C .
- D. 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. Make a 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 a mounted 5 μm FFPET section to a container with sufficient xylene to cover the tissue; soak for 5 minutes.
- B. Transfer the slide to a container with sufficient absolute ethanol to cover the tissue; soak for 5 minutes.
- C. Remove the slide from the ethanol and allow the section to air dry completely (5 to 10 minutes).
- D. Perform macro-dissection if the sample contains less than 10% tumor content by area.
- E. Label one 1.5 mL Safe-Lock microcentrifuge tube for each sample with the sample identification information.
- F. Add 180 μL **DNA TLB** to the 1.5-mL Safe-Lock microcentrifuge tube.
- G. Add 70 μL of reconstituted **PK** to the Safe-Lock tube containing **DNA TLB**.
- H. Scrape the tissue off the slide and into the Safe-Lock tube. Immerse the tissue in the **DNA TLB/PK** mixture.
- I. Continue with 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.*

NOTE: *If the sample contains less than 10% tumor content by area, the section must be mounted on a slide for macro-dissection and the procedure detailed in 'Deparaffinization of FFPET Sections Mounted on Slides' must be followed.*

- A. Place one 5-micron FFPET section into a 1.5 mL Safe-Lock microcentrifuge tube labeled with the sample identification information for each sample.
- B. Add 500 μL Xylene to the Safe-Lock tube containing the FFPET section.
- C. Mix well by vortexing for 10 seconds.
- D. Let the tube stand for 5 minutes at 15°C to 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 to 30°C .
- G. Centrifuge at $16,000 \times g$ to $20,000 \times g$ for 2 minutes. Remove the supernatant without disturbing the pellet. Discard the supernatant into chemical waste.

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- 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. 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 the tube open.
- NOTE: Make sure the ethanol is completely evaporated and the pellet is dry before proceeding to the next step.**
- NOTE: If needed, dry pellets can be stored up to 24 hours at 2°C to 8°C.**
- K. Resuspend the tissue pellet in 180 µL DNA Tissue Lysis Buffer (**DNA TLB**).
- L. Add 70 µL of reconstituted **PK**.
- M. Continue with Step A of the DNA Isolation procedure.

SAMPLE PREPARATION

DNA Isolation Procedure

NOTE: Process a negative control concurrently with the sample(s). Prepare the negative control by combining 180 µL DNA Tissue Lysis Buffer (DNA TLB) and 70 µL PK solution in a 1.5 mL Safe-Lock microcentrifuge tube labeled as NEG CT. The negative control should be processed following the same procedure as the samples.

- A. Vortex the tubes containing the sample/**DNA TLB/PK** mixture and the negative control mixture (**NEG CT**) for 30 seconds.
- NOTE: The tissue must be fully immersed in the DNA TLB/PK mixture.**
- B. Place tubes in the 56°C dry heat block and incubate for 60 minutes.
- C. Vortex the tubes for 10 seconds.
- NOTE: The tissue must be fully immersed in the DNA TLB/PK mixture.**
- D. Place tubes in the 90°C dry heat block and incubate for 60 minutes.
- NOTE: During the incubation, prepare the required number of filter tubes (FTs) with hinged caps by placing the FT onto a collection tube (CT) and labeling each FT cap with the proper sample or control identification.**
- NOTE: Each sample will need 1 FT, 3 CTs and 1 elution tube (1.5 mL microcentrifuge tube).**
- NOTE: During the incubation, label the required number of elution tubes (1.5 mL microcentrifuge tube) with the proper sample or control identification information.**
- E. Allow the tubes to cool to 15°C to 30°C. After cooling, pulse-centrifuge the tubes to collect liquid from the caps.
- F. Add 200 µL **DNA PBB** to each tube; mix by pipetting up and down 3 times.
- G. Incubate the tubes at 15°C to 30°C for 10 minutes.
- H. Add 100 µL isopropanol to each tube; mix lysate by pipetting up and down 3 times.
- I. Transfer each lysate into the appropriately labeled **FT/CT** unit.
- J. Centrifuge the **FT/CT** units at 8,000 x g for 1 minute.
- K. Place each **FT** onto a new **CT**. Discard the flow-through from the old **CT** into chemical waste, and properly dispose of the used **CT**.

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L. Add 500 µL working **WB I** to each **FT**.

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

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

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

O. Add 500 µL working **WB II** to each **FT**.

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

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

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

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

S. Place each **FT** into an elution tube (1.5 mL microcentrifuge tube) pre-labeled with sample or control identification. Discard the flow-through from the used **CT** into chemical waste, and properly dispose of the used **CT**.

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

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

V. Centrifuge the **FT** with elution tube at 8,000 x g for 1 minute to collect eluate into the elution tube. Properly dispose of the used **FT**.

W. Close the cap on the elution tube. The elution tube contains the DNA Stock. Proceed to Step A in the **DNA Quantitation** section.

NOTE: Measurement of DNA concentration 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.

B. Quantify DNA using 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 two consistent readings is necessary. The two measurements should be within $\pm 10\%$ of each other when the DNA concentration readings are ≥ 20.0 ng/µL. For DNA concentration readings < 20.0 ng/µL, the two measurements should be within ± 2 ng/µL. If the two measurements are not within $\pm 10\%$ of each other when the DNA concentration readings are ≥ 20.0 ng/µL or within ± 2 ng/µL when the DNA concentration readings are < 20.0 ng/µL, an additional 2 readings must be taken until the requirements are met. The average of these two new measurements should then be calculated.

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

C. The DNA Stock concentration from the samples must be ≥ 2 ng/µL to perform the **cobas**[®] PIK3CA Mutation Test. Three amplification/detections are run per sample, using 25 µL of a 2 ng/µL dilution of DNA Stock (total of 50 ng DNA) for each amplification/detection.

NOTE: Each DNA Stock must have a minimum concentration of 2 ng/µL to perform the cobas[®] PIK3CA Mutation Test. If the concentration of a DNA Stock is < 2 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 DNA TLB + PK, and perform DNA Isolation and Quantitation as described above. For unmounted samples, combine two sections into one tube and immerse the tissue in DNA TLB + PK, and perform DNA Isolation and Quantitation as described above. If the DNA Stock is still < 2 ng/µL, process a different FFPET sample block.

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

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AMPLIFICATION AND DETECTION

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

Instrument Set-Up:

Refer to the **cobas z 480** analyzer Instrument 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**[®] PIK3CA Mutation Test for detailed instructions on the PIK3CA workflow steps.

Dilution Calculation of Sample DNA Stock:

Dilution Calculation for DNA Stock Concentrations from 2 ng/μL to 36 ng/μL

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

NOTE: Three (3) amplification/detections are run for each sample requiring a total volume of 75 μL (25 μL for each of three reactions) of a 2 ng/μL dilution of DNA Stock (total of 150 ng DNA).

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

$$\mu\text{L of DNA stock} = (90 \mu\text{L} \times 2 \text{ ng}/\mu\text{L}) \div \text{DNA Stock concentration [ng}/\mu\text{L}]$$

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

$$\mu\text{L of DNA SD} = 90 \mu\text{L} - \mu\text{L of DNA Stock}$$

Example:

$$\text{DNA stock concentration} = 6.5 \text{ ng}/\mu\text{L}$$

$$\text{A. } \mu\text{L of DNA Stock} = (90 \mu\text{L} \times 2 \text{ ng}/\mu\text{L}) \div 6.5 \text{ ng}/\mu\text{L} = 27.7 \mu\text{L}$$

$$\text{B. } \mu\text{L of DNA SD} = (90 \mu\text{L} - 27.7 \mu\text{L}) = 62.3 \mu\text{L}$$

Dilution Calculation for DNA Stock Concentrations > 36 ng/μL

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

NOTE: Three (3) amplification/detections are run for each sample requiring a total volume of 75 μL (25 μL for each of three reactions) of a 2 ng/μL dilution of DNA stock (total of 150 ng DNA).

A. At DNA Stock concentrations > 36 ng/μL, use the following formula to calculate the amount of DNA Specimen diluent (**DNA SD**) required to prepare at least 90 μL of diluted DNA stock. This is to ensure that each sample uses a minimum of 5 μL of DNA stock.

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

$$\text{Vol. of DNA SD required in } \mu\text{L} = ((5 \mu\text{L of DNA stock} \times \text{DNA stock concentration in ng}/\mu\text{L}) / 2 \text{ ng}/\mu\text{L}) - 5 \mu\text{L}$$

Example:

$$\text{DNA stock concentration} = 100 \text{ ng}/\mu\text{L}$$

$$\text{A. Vol. of DNA SD required in } \mu\text{L} = ((5 \mu\text{L} \times 100 \text{ ng}/\mu\text{L}) / 2 \text{ ng}/\mu\text{L}) - 5 \mu\text{L} = 245 \mu\text{L}$$

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

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Sample Dilution

- A. Prepare the appropriate number of 1.5 mL Safe-Lock microcentrifuge tubes for DNA Dilutions by labeling them with the proper sample identification.
- B. Using a pipettor with an aerosol-resistant tip, pipette the calculated volumes of **DNA SD** into the respectively labeled tubes. Pipette 45 µL of **DNA SD** into a Safe-Lock tube labeled as **NEG CT**.
- C. Vortex each DNA stock and the negative control for 5 to 10 seconds.
- D. Using a pipettor with an aerosol-resistant pipette tip (new tip for each pipetting), gently pipette the calculated volume of each DNA stock into the respective tube containing **DNA SD**. Pipette 45 µL of negative control (extracted eluate) into the **NEG CT** tube.
- E. Cap the tubes and vortex each for 5 to 10 seconds.
- F. Change gloves.

Preparation of Working Master Mixes (MMX-1, MMX-2 and MMX-3)

NOTE: PIK3CA MMX-1, PIK3CA MMX-2, PIK3CA MMX-3, and working MMX are light-sensitive and must be protected from prolonged exposure to light.

NOTE: Due to the viscosity of the PIK3CA MIXES and working MMX, pipette slowly to ensure all mix is completely dispensed from the tip.

NOTE: The PIK3CA MMX-1, PIK3CA MMX-2, and PIK3CA MMX-3 may appear light blue/purplish. This does not affect the performance of the reagent.

Prepare three bulk working MMX, one containing **PIK3CA MMX-1**, one containing **PIK3CA MMX-2**, and the other containing **PIK3CA MMX-3** in separate 1.5 mL Safe-Lock microcentrifuge tubes.

- A. Calculate the volume of **PIK3CA MMX-1** or **PIK3CA MMX-2** or **PIK3CA MMX-3** required for each working MMX using the following formula:

$$\text{Volume of PIK3CA MMX-1 or PIK3CA MMX-2 or PIK3CA MMX-3 required} = (\text{Number of Samples} + 2 \text{ Controls} + 1) \times 20 \mu\text{L}$$

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

$$\text{Volume of MGAC required} = (\text{Number of Samples} + 2 \text{ Controls} + 1) \times 7 \mu\text{L}$$

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

Table 1
Volumes of Reagents Needed for Working MMX-1, Working MMX-2 and Working MMX-3

		# of Samples*									
		1	2	3	4	5	6	7	8	9	10
MMX	20 µL	80	100	120	140	160	180	200	220	240	260
MGAC	7 µL	28	35	42	49	56	63	70	77	84	91
Total Vol. for Each Working MMX (µL)		108	135	162	189	216	243	270	297	324	351

* Volumes for # of Samples is based on the sum of the # Samples + 2 Controls + 1

- C. Remove the appropriate number of **PIK3CA MMX-1, PIK3CA MMX-2, PIK3CA MMX-3, and MGAC** vials from 2°C to 8°C storage. Vortex each reagent for 5 seconds and collect liquid at the bottom of the tube before use. Label a sterile microcentrifuge tube for working MMX-1, working MMX-2, and working MMX-3.

- D. Add the calculated volume of **PIK3CA MMX-1** or **PIK3CA MMX-2** or **PIK3CA MMX-3** to their respective working MMX tube.

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- E. Add the calculated volume of **MGAC** to the working MMX tubes.
- F. Vortex the tubes for 3 to 5 seconds to ensure adequate mixing.

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

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

Preparation of Plate

Figure 1
Plate layout for the cobas® PIK3CA Mutation Test

Row / Column	1	2	3	4	5	6	7	8	9	10	11	12
A	MC MMX-1	MC MMX-2	MC MMX-3	S7 MMX-1	S7 MMX-2	S7 MMX-3	S15 MMX-1	S15 MMX-2	S15 MMX-3	S23 MMX-1	S23 MMX-2	S23 MMX-3
B	NEG MMX-1	NEG MMX-2	NEG MMX-3	S8 MMX-1	S8 MMX-2	S8 MMX-3	S16 MMX-1	S16 MMX-2	S16 MMX-3	S24 MMX-1	S24 MMX-2	S24 MMX-3
C	S1 MMX-1	S1 MMX-2	S1 MMX-3	S9 MMX-1	S9 MMX-2	S9 MMX-3	S17 MMX-1	S17 MMX-2	S17 MMX-3	S25 MMX-1	S25 MMX-2	S25 MMX-3
D	S2 MMX-1	S2 MMX-2	S2 MMX-3	S10 MMX-1	S10 MMX-2	S10 MMX-3	S18 MMX-1	S18 MMX-2	S18 MMX-3	S26 MMX-1	S26 MMX-2	S26 MMX-3
E	S3 MMX-1	S3 MMX-2	S3 MMX-3	S11 MMX-1	S11 MMX-2	S11 MMX-3	S19 MMX-1	S19 MMX-2	S19 MMX-3	S27 MMX-1	S27 MMX-2	S27 MMX-3
F	S4 MMX-1	S4 MMX-2	S4 MMX-3	S12 MMX-1	S12 MMX-2	S12 MMX-3	S20 MMX-1	S20 MMX-2	S20 MMX-3	S28 MMX-1	S28 MMX-2	S28 MMX-3
G	S5 MMX-1	S5 MMX-2	S5 MMX-3	S13 MMX-1	S13 MMX-2	S13 MMX-3	S21 MMX-1	S21 MMX-2	S21 MMX-3	S29 MMX-1	S29 MMX-2	S29 MMX-3
H	S6 MMX-1	S6 MMX-2	S6 MMX-3	S14 MMX-1	S14 MMX-2	S14 MMX-3	S22 MMX-1	S22 MMX-2	S22 MMX-3	S30 MMX-1	S30 MMX-2	S30 MMX-3

Where: NEG = Negative Control, MC = PIK3CA Mutant Control, S# = sample ID, and MMX-# corresponds to Master Mix Reagent 1, 2, or 3.

NOTE: Any given sample must be spread across three consecutive columns in one row in order to generate a result.

- A. Pipette 25 µL of working MMX into each reaction well of the microwell plate (AD-plate) that is needed for the run. Do not allow the pipettor tip to touch the plate outside the well.
- Add working MMX-1 to the microwell plate (AD-plate) wells in columns 1, 4, 7, and 10, as needed.
 - Add working MMX-2 to the microwell plate (AD-plate) wells in columns 2, 5, 8, and 11, as needed.
 - Add working MMX-3 to the microwell plate (AD-plate) wells in columns 3, 6, 9, and 12, as needed.
- B. Pipette 25 µL of **PIK3CA MC** into wells **A1**, **A2**, and **A3** of the microwell plate (AD-plate); mix well using pipette to aspirate and dispense within the well a minimum of two times.
- C. Using a new pipettor tip, pipette 25 µL of **NEG CT** into wells **B1**, **B2**, and **B3** of the microwell plate (AD-plate); mix well using pipette to aspirate and dispense within the well a minimum of two times.

NOTE: Each run must contain positive control (PIK3CA MC) in wells A1, A2 and A3 ,and negative control (NEG CT) in wells B1, B2, and B3 or the run will be invalidated by the PIK3CA Analysis Package Software.

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

- D. Using new pipettor tips for each diluted sample DNA, add 25 uL of the first sample DNA to wells C1, C2, and C3 of the microwell plate (AD-plate); mix well using pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for the diluted DNA from the second sample (wells D1, D2 and D3). Follow the template in Figure 1 until all samples' DNA Dilutions are loaded onto the microwell plate (AD-plate).
- E. Cover the microwell plate (AD-plate) with sealing foil (supplied with the plates). Use the sealing foil applicator to seal the foil firmly to the microwell plate (AD-plate).
- F. Confirm that all liquid is collected at the bottom of each well before starting PCR.

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

Starting PCR

Refer to the **cobas**[®] PIK3CA Operator's Manual for detailed instructions on the PIK3CA workflow steps.

INTERPRETATION OF RESULTS

NOTE: All run and sample validation is performed by the cobas[®] 4800 software.

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

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

**Table 2
Result Interpretation of cobas[®] PIK3CA Mutation Test**

Test Result	Mutation Result	Interpretation
Mutation Detected	R88Q N345K C420R E542K E545X (E545A, E545D [‡] , E545G, or E545K) Q546X (Q546E, Q546K, Q546L, or Q546R) M1043I [§] H1047X (H1047L, H1047R, or H1047Y) G1049R (More than one mutation may be present)	Mutation detected in specified targeted PIK3CA region.
Mutation Not Detected*	N/A	Mutation not detected in targeted PIK3CA regions.
Invalid	N/A	Sample result is invalid. Repeat the testing of samples with invalid results following the instructions outlined in the “Retesting of Samples with Invalid Results” section below. For a list of result flags including flag descriptions, refer to the cobas [®] 4800 system Operator’s Manual for cobas [®] PIK3CA Mutation Test (For Research Use Only).
Failed	N/A	Failed run due to hardware or software failure. Contact your local Roche office for technical assistance.

* A Mutation Not Detected result does not preclude the presence of a mutation in the targeted PIK3CA regions because results depend on percent mutant sequences, adequate sample integrity, absence of inhibitors, and sufficient DNA to be detected.

[‡] For the E545D amino acid change, only the nucleotide change c.1635G>T mutation is detected by the test.

[§] For the M1043I amino acid change, only the nucleotide change c.3129G>T mutation is detected by the test.

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Retesting of Samples with Invalid Results

- A. Repeat dilution of the invalid sample DNA stock starting from "**Dilution Calculation of Sample DNA Stock**" and "**Sample Dilution**" procedures in the "**AMPLIFICATION and DETECTION**" section.
- B. After performing the DNA stock dilution to 2 ng/μL described in "**Sample Dilution**" continue with "**Preparation of Working Master Mix (working MMX)**" and the remainder of the amplification and detection procedure.

NOTE: *If the sample remains invalid after retesting or there was not enough DNA stock to prepare another dilution in Retesting of Samples with Invalid Results, step A, repeat the entire test procedure for that sample, starting with Deparaffinization and DNA Isolation using a new 5-micron FFPE section.*

QUALITY CONTROL

One set of PIK3CA Mutant Control (**PIK3CA MC**) and negative control (**NEG CT**) for working MMX-1, working MMX-2, and working MMX-3 are included in each run of up to 30 samples. A run is valid if the **PIK3CA MC** wells (**A1, A2** and **A3**), and the **NEG CT** wells (**B1, B2** and **B3**) are valid. If the **PIK3CA MC** or **NEG CT** for working MMX-1 or working MMX-2 or working MMX-3 are invalid, the entire run is invalid and must be repeated. Prepare a fresh dilution of the previously isolated sample DNA Stock to set up a new microwell plate (AD-plate) with controls for amplification and detection.

Positive Control

The **PIK3CA MC** result must be 'Valid' for working MMX-1, working MMX-2, and working MMX-3. If the **PIK3CA MC** results are consistently invalid, contact your local Roche office for technical assistance.

Negative Control

The **NEG CT** result must be 'Valid' for working MMX-1, working MMX-2, and working MMX-3. If the **NEG CT** results are consistently invalid, contact your local Roche office for technical assistance.

PROCEDURAL PRECAUTIONS

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

PROCEDURAL LIMITATIONS

1. The **cobas**[®] PIK3CA Mutation Test was verified with breast cancer FFPE samples.
2. The **cobas**[®] PIK3CA Mutation Test was verified using the **cobas**[®] DNA Sample Preparation Kit (Roche P/N: 05985536190).
3. Detection of a mutation is dependent on the number of copies present in the sample and may be affected by sample integrity, amount of isolated DNA, and the presence of interfering substances.
4. Reliable results are dependent on adequate sample fixation, transport, storage and processing. Follow the procedures in this Package Insert and in the **cobas**[®] 4800 system Operator's Manual Software Version 2.0 for **cobas**[®] PIK3CA Mutation Test.
5. The effects of other potential variables such as sample fixation variables have not been evaluated.
6. The addition of AmpErase enzyme into the **cobas**[®] PIK3CA Mutation Test 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.
7. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas**[®] 4800 system.

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8. Only the **cobas z** 480 analyzer was verified for use with this product. No other thermal cycler with real-time optical detection can be used with this product.
9. 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.
10. Though rare, mutations within the regions of the genomic DNA of the PIK3CA gene covered by the **cobas**[®] PIK3CA Mutation Test's primers and/or probes may result in failure to detect the presence of a mutation.
11. The presence of PCR inhibitors may cause false negative or invalid results.
12. Though rare, the **cobas**[®] PIK3CA Mutation Test shows some limited cross-reactivity (results of "Mutation Detected") for mutations flanking the targeted mutations in exons 9 and 20.
13. The **cobas**[®] PIK3CA Mutation Test was verified for use with 50 ng of DNA per reaction well. DNA input amounts lower than 50 ng per reaction well are not recommended.
14. Though rare, samples containing nearby double mutations on the same chromosome may interfere with the detection of one of the two mutations.

ANALYTICAL PERFORMANCE

The following data demonstrate the analytical performance of the **cobas**[®] PIK3CA Mutation Test. The data are not intended to demonstrate any clinical performance claims for the test. The **cobas**[®] PIK3CA Mutation Test is for Research Use Only and is not intended for diagnostic procedures.

Analytical Sensitivity Using FFPET Sample Blends

DNA isolated from four breast cancer FFPET samples with PIK3CA mutations (three samples with exon 9 mutations and one sample with an exon 20 mutation) were each blended with DNA isolated from PIK3CA wild-type breast cancer FFPET samples to achieve sample DNA blends targeting 5.0% mutation level as determined by a massively parallel pyrosequencing method, 454 sequencing (454 Life Sciences, a Roche company, Branford, CT, USA). Serial dilutions of each sample DNA blend were prepared and a total of twenty (20) replicates of each panel member were run using 2 **cobas**[®] PIK3CA Mutation Test kit lots (n=20/panel member). The analytical sensitivity of each sample was determined by the lowest amount of DNA that gave a PIK3CA "Mutation Detected" rate of at least 95% for the targeted mutation, shown in Table 3.

Table 3
Analytical Sensitivity of the **cobas[®] PIK3CA Mutation Test using FFPET Sample DNA Blends**

PIK3CA Exon	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	Percent Mutation*	Amount of DNA (ng/25 µL) to achieve ≥ 95% "Mutation Detected" Rate (N=20)
9	E542K	1624 G>A	5.5%	1.5 ng
	E545K	1633 G>A	4.7%	5.0 ng
	Q546K	1636 C>A	4.4%	15.0 ng
20	H1047R	3140 A>G	4.8%	1.5 ng

* Percent mutation determined by 454 sequencing.

The **cobas**[®] PIK3CA Mutation Test was able to detect mutations in PIK3CA exons 9 and 20 with approximately 5% mutation level using the standard DNA input of ranging from 1.5 ng to 15.0 ng per reaction well. This would indicate that the test will detect the tested PIK3CA mutations when ~70% to ~97% of the DNA is degraded or non-amplifiable due to the fixation process.

Inclusivity with Plasmids

For each of the seventeen PIK3CA mutations targeted by the **cobas**[®] PIK3CA Mutation Test, a DNA plasmid construct was blended with wild-type cell line DNA to a targeted final composition of 5% mutant sequence based on genomic DNA equivalents. A total of at least 20 replicates for each plasmid blend was tested with a DNA input of 50 ng using 2 **cobas**[®] PIK3CA Mutation Test kit lots. The observed "Mutation Detected" rates for each 5% plasmid blend are shown in Table 4.

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Table 4
Mutations Detected by the cobas® PIK3CA Mutation Test Using 5% Mutant Plasmid DNA Blends

PIK3CA Exon	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	Cosmic ID	"Mutation Detection" Rate at a target of 5% Mutant DNA and 50 ng DNA Input (N ≥ 20)
1	R88Q	263 G>A	746	100%
4	N345K	1035 T>A	754	95%
7	C420R	1258 T>C	757	100%
9	E542K	1624 G>A	760	100%
	E545A	1634 A>C	12458	100%
	E545D	1635 G>T	765	100%
	E545G	1634 A>G	764	100%
	E545K	1633 G>A	763	100%
	Q546E	1636 C>G	6147	100%
	Q546K	1636 C>A	766	100%
	Q546L	1637 A>T	25041	100%
	Q546R	1637 A>G	12459	100%
20	M1043I	3129 G>T	773	100%
	H1047L	3140 A>T	776	100%
	H1047R	3140 A>G	775	100%
	H1047Y	3139 C>T	774	100%
	G1049R	3145 G>C	12597	100%

Correlation to 2X Bi-directional Sanger Sequencing and 454 Sequencing

Comparison testing of 110 breast cancer FFPE specimens using each of 2 lots of the **cobas®** PIK3CA Mutation Test and 2X-Bi-directional sequencing (Sanger) was performed to determine positive, negative and overall percent agreement between methods. Discordant results between the **cobas®** PIK3CA Mutation Test and Sanger were tested using 454 sequencing to resolve discordance.

The comparison of the 110 valid results for Sanger and the **cobas®** PIK3CA Mutation Test is shown in Table 5. Note that lot 1 and lot 2 of the **cobas®** PIK3CA Mutation Test produced the same test results for each of the 110 breast cancer FFPE specimens.

Table 5
cobas PIK3CA Mutation Test vs. 2X Bi-Directional Sequencing

		Sanger Sequencing		
		MD	MND	Total
cobas® PIK3CA Mutation Test	MD	50*	4	54
	MND	0	56	56
	Total	50	60	110

Positive agreement = 100% (95% CI = 92.9 - 100%)

Negative agreement = 93.3% (95% CI = 84.1 - 97.4%)

Overall agreement = 96.4% (95% CI = 91.0 - 98.6%)

MD: Mutation Detected

MND: Mutation Not Detected

* One specimen was MD by both Sanger sequencing and the **cobas®** PIK3CA Mutation Test, but Sanger sequencing missed a second mutation (see Table 7).

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The comparison between the **cobas**[®] PIK3CA Mutation Test and Sanger evaluated nine targets for each sample. A total of 990 calls were made based on the results of the 110 valid samples. Table 6 shows the comparison of the **cobas**[®] PIK3CA Mutation Test and Sanger on a per call basis.

Table 6
Per Call Comparison of the cobas[®] PIK3CA Mutation Test vs. Sanger

		Sanger Sequencing										
		R88Q	N345K	C420R	E542K	E545X	Q546X	M1043I	H1047X	G1049R	MND	Total
cobas [®] PIK3CA Mutation Test	R88Q	-	-	-	-	-	-	-	-	-	-	0
	N345K	-	4	-	-	-	-	-	-	-	-	4
	C420R	-	-	3	-	-	-	-	-	-	-	3
	E542K	-	-	-	5	-	-	-	-	-	2	7
	E545X	-	-	-	-	10	-	-	-	-	-	10
	Q546X	-	-	-	-	-	4	-	-	-	-	4
	M1043I	-	-	-	-	-	-	-	-	-	-	0
	H1047X	-	-	-	-	-	-	-	22	-	3	25
	G1049R	-	-	-	-	-	-	-	-	2	-	2
	MND	-	-	-	-	-	-	-	-	-	935	935
	Total	0	4	3	5	10	4	0	22	2	935	990

Discordant Analysis by 454 Sequencing

Five sample results were discordant between Sanger sequencing and the **cobas**[®] PIK3CA Mutation Test. Samples that gave discordant results between the **cobas**[®] PIK3CA Mutation Test and Sanger were analyzed by 454 sequencing and are shown in Table 7. A revised agreement analysis was performed based on the 454 sequencing results. In this analysis, specimens with 454 sequencing results that agreed with the **cobas**[®] PIK3CA Mutation Test result were considered concordant.

Table 7
Discordant Specimen Resolution by 454 Sequencing

Sample	Sanger	cobas [®] PIK3CA Mutation Test Lot 1	cobas [®] PIK3CA Mutation Test Lot 2	454 Sequencing Resolution
Sample 1*	MND	H1047X	H1047X	H1047R (3.7% mutation)
Sample 2*	MND	E542K	E542K	E542K (4.3% mutation)
Sample 3*	MND	H1047X	H1047X	H1047R (2.6% mutation)
Sample 4**	C420R	C420R;H1047X	C420R;H1047X	C420R (18.4% mutation) H1047R (1.3% mutation)
Sample 5***	MND	E542K	E542K	E542K (9.2% mutation) H1047R (1.8% mutation)

* Samples 1, 2, and 3 were confirmed by 454 sequencing to be PIK3CA mutants with < 5.0% mutation.

** Sample 4 was confirmed by 454 sequencing to be a PIK3CA double mutant (1.3% H1047R mutation and 18.4% C420R mutation).

*** Sample 5 was confirmed by 454 sequencing to be an E542K mutant with 9.2% mutation. In addition, 454 sequencing identified a second low level H1047R mutant (1.8%) that was not detected by either Sanger sequencing or the **cobas**[®] PIK3CA Mutation Test.

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After the **cobas**[®] PIK3CA Mutation Test vs. Sanger discordant samples were resolved by 454 sequencing, the overall concordance of the **cobas**[®] PIK3CA Mutation Test with sequencing was 100% across all targeted mutations as shown in Table 8.

Table 8
Agreement Analysis of the cobas[®] PIK3CA Mutation Test vs. Sanger with Discordant Resolution by 454 Sequencing

		Sanger Sequencing, Resolved with 454 Sequencing		
		MD	MND	Total
cobas [®] PIK3CA Mutation Test	MD	54	0	54
	MND	0	56	56
	Total	55	56	110

MD agreement = 100% (95% CI = 93.4- 100%)

MND agreement = 100% (95% CI = 93.6 - 100%)

Overall agreement = 100% (95% CI = 96.6- 100%)

Table 9 shows the comparison of the **cobas**[®] PIK3CA Mutation Test and Sanger with discordant resolution by 454 sequencing on a per call basis.

Table 9
Per Call Comparison of the cobas[®] PIK3CA Mutation Test vs. Sanger with Discordant Resolution by 454 Sequencing

		Sanger Sequencing, Resolved with 454 Sequencing										
		R88Q	N345K	C420R	E542K	E545X	Q546X	M1043I	H1047X	G1049R	MND	Total
cobas [®] PIK3CA Mutation Test	R88Q	-	-	-	-	-	-	-	-	-	-	0
	N345K	-	4	-	-	-	-	-	-	-	-	4
	C420R	-	-	3	-	-	-	-	-	-	-	3
	E542K	-	-	-	7	-	-	-	-	-	-	7
	E545X	-	-	-	-	10	-	-	-	-	-	10
	Q546X	-	-	-	-	-	4	-	-	-	-	4
	M1043I	-	-	-	-	-	-	-	-	-	-	0
	H1047X	-	-	-	-	-	-	-	25	-	-	25
	G1049R	-	-	-	-	-	-	-	-	2	-	2
	MND	-	-	-	-	-	-	-	-	-	935	935
	Total	0	4	3	7	10	4	0	25	2	935	990

Repeatability

Repeatability of the **cobas**[®] PIK3CA Mutation Test was assessed using six FFPET specimens, including: 2 wild-type specimens; 4 mutant specimens each with one E542K, E545K, Q546K, or H1047R respective mutation. These specimens were tested in duplicate by two operators, using two different reagent lots and two **cobas z** 480 analyzers over 8 days. The **cobas**[®] PIK3CA Mutation Test had a correct call rate of 99.5% (191/192).

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Catalogue Number



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