cobas® PIK3CA Mutation Test

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

cobas® PIK3CA Mutation Test	**PIK3CA**	24 Tests	M/N: 06523013190

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

PRINCIPLES OF THE PROCEDURE

The **cobas**® PIK3CA Mutation Test (**cobas** PIK3CA 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 tissue (FFPET).

The **cobas** PIK3CA 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 samples 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 Test kit.

PCR Amplification

Target Selection

The **cobas** PIK3CA 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.

Automated Real-time Mutation Detection

The **cobas** PIK3CA 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 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 55ºC, i.e., throughout the thermal cycling steps, and therefore does not destroy target amplicon.

REAGENTS

cobas® PIK3CA Mutation Test (M/N: 06523013190)	**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.

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

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

K. 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.

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-A3[3].

M. **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.

N. Wear eye protection, laboratory coats, and disposable gloves when handling any reagents. Avoid contact of these materials with the skin, eyes, or mucous membranes. If contact does occur, immediately wash with large amounts of water. Burns can occur if left untreated. If spills occur, dilute with water before wiping dry.

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

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

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

R. To reduce the risk of contamination, samples and/or reagents should not be transferred from one microwell plate to another microwell plate.

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

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

STORAGE AND HANDLING REQUIREMENTS

A. Do not freeze reagents.

B. Store **MGAC, PIK3CA MMX-1, PIK3CA MMX-2, PIK3CA MMX-3, PIK3CA MC,** and **DNA SD** at 2ºC to 8ºC. Once opened, these reagents are stable for 4 uses within 90 days or until the expiration date, whichever comes first.

C. **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.

D. 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.

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E. Processed specimens (extracted DNA) are stable for up to 24 hours at 15ºC to 30°C, up to 14 days at 2ºC 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.

F. 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

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

* *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.*

Instrumentation and Software

- **cobas z** 480 analyzer
- **cobas**® 4800 SR2 System Control Unit with OS XP or OS Win7 image
- **cobas**® 4800 SR2 System Software version 2.0 or higher
- PIK3CA Analysis Package Software (RUO) version 1.0 or higher
- Barcode Reader (Roche M/N 05339910001)
- Printer HP P2055d (Roche M/N 05704375001)

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 Test.

B. Sample Transport

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

5 µm sections may be stored at 15ºC 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 that 10% tumor content by area should be macro-dissected after deparaffinization.

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

Run Size

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 Test kits of the same lot will be required.

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

Workflow

The **cobas** PIK3CA 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 Test kit.

DNA Isolation

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

Macro-dissection

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

DNA Quantitation:

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

NOTE: Store DNA stock according to instructions in Sample Collection, Storage and Transport section.

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

B. Quantify DNA using a spectrophotometer according to the manufacturer's protocol. Use **DNA EB** from the **cobas®** DNA Sample Preparation Kit as the blank for the instrument. An average of two consistent readings is necessary. The two measurements should be within ±10% of each other when the DNA concentration readings are $\geq$ 20.0 ng/µL. For DNA concentration readings < 20.0 ng/µL, the two measurements should be within ± 2 ng/µL. If the two measurements are not within ± 10% of each other when the DNA concentration readings are $\geq$ 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 $\geq$ 2 ng/µL to perform the **cobas** PIK3CA 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 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 TLB + PK from the cobas® DNA Sample Preparation Kit, and perform DNA isolation and quantitation. For unmounted samples, combine two sections into one tube and perform deparaffinization, DNA isolation and quantitation. If the DNA Stock is still < 2 ng/µL, process a different FFPET sample block.

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

Test Order Set-up:

Refer to the **cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance for **cobas** PIK3CA 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:

   µL of DNA stock = (90 µL x 2 ng/µL) ÷ DNA Stock concentration [ng/µL]

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

   µL of **DNA SD** = 90 µL - µL of DNA Stock

Example:

DNA stock concentration = 6.5 ng/µL

A. µL of DNA Stock = (90 µL x 2 ng/µL) ÷ 6.5 ng/µL = 27.7 µL
B. µL of **DNA SD** = (90 µL - 27.7 µL) = 62.3 µL

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:

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

Example:

DNA stock concentration = 100 ng/µL

A. Vol. of **DNA SD** required in µL = ((5 µL x 100 ng/µL) / 2 ng/µL) - 5 µL = 245 µL
B. Use the calculated volume of **DNA SD** to dilute 5 µL of DNA stock.

Sample Dilution

A. Prepare the appropriate number of 1.5 mL 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 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 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:

Volume of **PIK3CA MMX-1** or **PIK3CA MMX-2** or **PIK3CA MMX-3** required = (Number of Samples + 2 Controls +1) x 20 µL

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

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

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

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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.

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 and controls should be added to the AD-plate within 1 hour after the preparation of the working MMXs.

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

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.

PCR Set-up

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

- Add working MMX-1 to the AD-plate wells in columns 1, 4, 7, and 10, as needed.
- Add working MMX-2 to the AD-plate wells in columns 2, 5, 8, and 11, as needed.
- Add working MMX-3 to the 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 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 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.

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 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 AD-plate.

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

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**® 4800 System – Operator's Manual or **cobas**® 4800 – User Assistance for **cobas** PIK3CA Test 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 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 or No Mutation Detected*	N/A	No mutation 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) or **cobas®** 4800 System – User Assistance.
Failed	N/A	Failed run due to hardware or software failure. Contact your local Roche office for technical assistance.

** A Mutation Not Detected or No Mutation Detected result does not preclude the presence of a mutation in the 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.

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, obtain a new 5-µm FFPET section of tissue and re-isolate DNA using the cobas® DNA Sample Preparation Kit (M/N 05985536190) and repeat testing.

QUALITY CONTROL

One 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. 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 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 Test was verified with breast cancer FFPET samples.
2. The **cobas** PIK3CA Test was verified using the **cobas®** DNA Sample Preparation Kit (Roche M/N: 05985536190).
3. Detection of a mutation is dependent on the number of copies present in the 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 the **cobas®** DNA Sample Preparation Kit Instructions for Use (M/N 05985536190), in this instructions for use, and in the **cobas®** 4800 System – Operator's Manual or **cobas®** 4800 System - User Assistance.
5. The effects of other potential variables such as sample fixation variables have not been evaluated.
6. The addition of AmpErase enzyme into the **cobas** PIK3CA Test Master Mix enables selective amplification of target DNA; however, good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary to avoid contamination of reagents
7. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas®** 4800 system.
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.

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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 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 Test shows some limited cross-reactivity (results of "Mutation Detected") for mutations flanking the targeted mutations in exons 9 and 20 (e.g., E545K with a high mutant percentage may return a mutation result of E545X;Q546X).

13. The **cobas** PIK3CA 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 DNA strand may interfere with the detection of one of the two mutations (e.g., P539R (CCT>CGT) may interfere with the detection of E542K or Y343Y (TAC>TAT) may interfere with the detection of N345K).

ANALYTICAL PERFORMANCE

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

Limit of Detection Using FFPET Sample Blends

DNA isolated from 33 breast cancer FFPET samples with PIK3CA mutations were blended with DNA isolated from 25 PIK3CA wild-type breast cancer FFPET samples to achieve 42 unique DNA blends targeting 10.0%, 7.5%, 5.0%, 2.5%, and 1.0% mutation levels as determined by 454 sequencing, a massively parallel pyrosequencing method. Dilutions of each sample DNA blend were prepared and a total of twenty-one (21) replicates of each mutation level were run using 3 **cobas** PIK3CA Test kit lots (n=21/panel member). The limit of detection for each sample was determined by the lowest percent mutation that gave a PIK3CA "Mutation Detected" rate of at least 95% for the targeted mutation, shown in Table 3.

Table 3
Limit of Detection for the cobas PIK3CA Test using FFPET Sample DNA Blends

PIK3CA Exon	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	Cosmic ID	Sample	Percent Mutation in the Panel Member to achieve ≥95% "Mutation Detected" Rate with 50 ng DNA input per reaction well (N=21 replicates)
1	R88Q	263 G>A	746	Sample 1	2.2%
				Sample 2	1.3%
				Sample 3	1.1%
4	N345K	1035 T>A	754	Sample 4	2.2%
				Sample 5	1.9%
				Sample 6	1.3%
7	C420R	1258 T>C	757	Sample 7	1.7%
				Sample 8	1.9%
				Sample 9	1.6%
9	E542K	1624 G>A	760	Sample 10	1.1%
				Sample 11	1.2%
				Sample 12	1.1%

PIK3CA Exon	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	Cosmic ID	Sample	Percent Mutation in the Panel Member to achieve ≥95% "Mutation Detected" Rate with 50 ng DNA input per reaction well (N=21 replicates)
	E545A	1634 A>C	12458	Sample 13	2.8%
				Sample 14	0.9%
				Sample 15	1.6%
	E545G	1634 A>G	764	Sample 16	1.8%
				Sample 17	1.2%
				Sample 18	1.6%
	E545K	1633 G>A	763	Sample 19	3.3%
				Sample 20	1.5%
				Sample 21	1.8%
	Q546E	1636 C>G	6147	Sample 22	3.5%
				Sample 23	1.6%
				Sample 24	2.5%
9	Q546K	1636 C>A	766	Sample 25	3.4%
				Sample 26	2.3%
				Sample 27	2.7%
	Q546R	1637 A>G	12459	Sample 28	1.5%
				Sample 29	3.2%
				Sample 30	1.3%
20	H1047L	3140 A>T	776	Sample 31	2.8%
				Sample 32	1.8%
				Sample 33	3.3%
	H1047R	3140 A>G	775	Sample 34	2.8%
				Sample 35	1.5%
				Sample 36	1.0%
	H1047Y	3139 C>T	774	Sample 37	3.5%
				Sample 38	2.2%
				Sample 39	3.4%
	G1049R	3145 G>C	12597	Sample 40	1.0%
				Sample 41	0.7%
				Sample 42	1.0%

The **cobas** PIK3CA Test was able to detect targeted mutations in the PIK3CA gene with a percent mutation level ranging from 0.7% to 3.5% with a 50 ng/PCR DNA input.

Detection of Rare Genotypes using Plasmids

For the three PIK3CA mutations listed in Table 4, a DNA plasmid construct was blended with wild-type DNA to prepare low percent mutant DNA samples. A total of at least 20 replicates for each plasmid blend was tested with a DNA input of 50 ng using at least one **cobas** PIK3CA Test kit lot. The binomial 95% confidence limits for each plasmid blend are shown in Table 4.

Table 4
Mutations Detected by the cobas PIK3CA Test Using Mutant Plasmid DNA Blends

PIK3CA Exon	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	Cosmic ID	Actual Percent Mutation	Binomial 95% lower confidence limit (N ≥ 20)	Binomial 95% upper confidence limit (N ≥ 20)
9	E545D	1635 G>T	765	1.2%	62%	97%
9	Q546L	1637 A>T	25041	2.1%	83%	100%
20	M1043I	3129 G>T	773	2.6%	83%	100%

Correlation to Reference Method

Comparison testing of 206 breast cancer FFPET samples using each of 2 lots of the **cobas** PIK3CA 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 Test and Sanger sequencing were tested using 454 sequencing to resolve discordance.

cobas PIK3CA Test and Sanger Sequencing Results

The comparison of the 205 valid results for Sanger sequencing and the **cobas** PIK3CA Test is shown in Table 5.

Table 5
cobas PIK3CA Test vs. 2X Bi-Directional Sequencing (Sanger)

		Sanger Sequencing		
		MD	**NMD**	**Total**
cobas PIK3CA Test	**MD**	95*	7	102
	NMD	0	103	103
	Invalid	0	1	1
	Total	95	111	206

Positive agreement = 100% (95% CI = 96.1 - 100%)

Negative agreement = 93.6% (95% CI = 87.4 - 96.9%)

Overall agreement = 96.6% (95% CI = 93.1 - 98.3%)

MD: Mutation Detected

NMD: No Mutation Detected

* *Five samples were MD by both Sanger sequencing and the **cobas** PIK3CA Test, but Sanger sequencing missed a second mutation (see Table 7).*

The comparison between the **cobas** PIK3CA Test and Sanger sequencing evaluated nine targets for each sample. A total of 1845 calls were made based on the results of the 205 valid samples. Table 6 shows the comparison of the **cobas** PIK3CA Test and Sanger sequencing on a per call basis.

Table 6
Per Call Comparison of the cobas PIK3CA Test vs. Sanger Sequencing

cobas PIK3CA Test (Lot 1)	R88Q	N345K	C420R	E542K	E545X	Q546X	M1043I	H1047X	G1049R	NMD	Total
R88Q	1	–	–	–	–	–	–	–	–	–	1
N345K	–	7	–	–	–	–	–	–	–	–	7
C420R	–	–	3	–	–	–	–	–	–	–	3
E542K	–	–	–	14	–	–	–	–	–	2	16
E545X	–	–	–	–	17	–	–	–	–	2	19
Q546X	–	–	–	–	–	8	–	–	–	1	9
M1043I	–	–	–	–	–	–	–	–	–	–	0
H1047X	–	–	–	–	–	–	–	42	–	7	49
G1049R	–	–	–	–	–	–	–	–	3	–	3
NMD	–	–	–	–	–	–	–	–	–	1738	1738
Invalid	–	–	–	–	–	–	–	–	–	9	9
Total	1	7	3	14	17	8	0	42	3	1759	1854

Discordant Analysis by 454 Sequencing

Seven sample test results were discordant between Sanger sequencing and the **cobas** PIK3CA Test. Five more samples were concordant for one mutation, however the **cobas** PIK3CA Test detected one additional mutation each. These twelve samples 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, samples with 454 sequencing results that agreed with the **cobas** PIK3CA Test result were considered concordant.

Table 7
Discordant Result Resolution by 454 Sequencing

Sample	Sanger	cobas PIK3CA Test Lot 1	454 Sequencing Resolution	cobas PIK3CA Test Lot 2	454 Sequencing Resolution
Sample 1	NMD	H1047X	H1047R (3.4% mutation)	H1047X	H1047R (2.5% mutation)
Sample 2	NMD	E542K	E542K (4.8% mutation)	E542K	E542K (3.4% mutation)
Sample 3	NMD	H1047X	H1047R (2.0% mutation)	H1047X	H1047R (2.8% mutation)
Sample 4	NMD	E542K	E542K (10.1% mutation)	E542K	E542K (8.3% mutation)
Sample 5	NMD	E545X	E545K (4.3% mutation)	E545X	E545K (2.2% mutation)
Sample 6	NMD	H1047X	H1047R (5.1% mutation) H1047Y (1.1% mutation)	H1047X	H1047R (4.1% mutation)
Sample 7	NMD	E545X	E545K (17.2% mutation)	E545X	E545K (25.6% mutation)
Sample 8*	H1047L	H1047X;Q546X	Q546K (2.2% mutation)	H1047X	N/A
Sample 9*	Q546R	H1047X;Q546X	H1047R (0.6% mutation) H1047Y (0.4% mutation)	Q546X	N/A
Sample 10	C420R	H1047X;C420R	H1047R (0.9% mutation)	H1047X;C420R	H1047R (1.1% mutation)
Sample 11	E545K	H1047X;E545X	H1047R (1.7% mutation)	H1047X;E545X	H1047R (1.8% mutation)
Sample 12	Q546E	H1047X;Q546X	H1047R (6.7% mutation)	H1047X;Q546X	H1047R (5.4% mutation)

* Samples 8 and 9 had discordant results between Lot 1 and Lot 2. Lot 2 results were concordant with Sanger sequencing, therefore no resolution testing was necessary.

NOTE: The cobas results for samples 8 – 12 detected the same PIK3CA mutations as Sanger sequencing, however additional mutations were detected and confirmed by 454 sequencing.

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

Table 8

Agreement Analysis of the cobas PIK3CA Test vs. Sanger Sequencing with Discordant Resolution by 454 Sequencing

		Sanger Sequencing, Resolved with 454 Sequencing		
		MD	**NMD**	**Total**
cobas PIK3CA Test	**MD**	102	0	102
	NMD	0	103	103
	Invalid	0	1	1
	Total	102	104	206

Positive agreement = 100% (95% CI = 96.4 - 100%)

Negative agreement = 100% (95% CI = 96.4 - 100%)

Overall agreement = 100% (95% CI = 98.2 - 100%)

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

Table 9

Per Call Comparison of the cobas PIK3CA Test vs. Sanger Sequencing with Discordant Resolution by 454 Sequencing

Cobas PIK3CA Test (Lot 1)	R88Q	N345K	C420R	E542K	E545X	Q546X	M1043I	H1047X	G1049R	NMD	Total
R88Q	1	-	-	-	-	-	-	-	-	-	1
N345K	-	7	-	-	-	-	-	-	-	-	7
C420R	-	-	3	-	-	-	-	-	-	-	3
E542K	-	-	-	16	-	-	-	-	-	-	16
E545X	-	-	-	-	19	-	-	-	-	-	19
Q546X	-	-	-	-	-	9	-	-	-	-	9
M1043I	-	-	-	-	-	-	-	-	-	-	0
H1047X	-	-	-	-	-	-	-	49	-	-	49
G1049R	-	-	-	-	-	-	-	-	3	-	3
NMD	-	-	-	-	-	-	-	-	-	1738	1738
Invalid	-	-	-	-	-	-	-	-	-	9	9
Total	1	7	3	16	19	9	0	49	3	1747	1854

Cross-Reactivity

The following non-targeted mutations were tested using plasmids for cross-reactivity at an approximate 50% input level: M1043I$_A$, M1043V, M1043T, G1049S, G1049A, E542V, E542Q, E545D$_C$, E545V, E545Q, Q546P, Q546H, and the PIK3CA pseudogene. These non-targeted mutations did not cross react (or interfere) with the **cobas** PIK3CA Test when added to samples containing wild-type and mutant PIK3CA sequences.

Evaluation of Potentially Interfering Substances

Endogenous

Triglycerides (≤ 37mM, CLSI recommended high concentration[6]) and hemoglobin (≤ 2 mg/mL, CLSI recommended high concentration[6]) have been shown not to interfere with the **cobas** PIK3CA Test when the substance was added to samples during the sample preparation procedure.

Samples with up to 90% adipose tissue and 70% necrotic tissue have been shown not to interfere with the **cobas** PIK3CA Test.

Exogenous

The following drugs were tested for interference at 3x C_{max}: Letrozole, Anastrozole, Capecitabine, Tamoxifen, Exemestane, Everolimus, Paclitaxel, Docetaxel, Cyclophosphamide, Doxorubicin, and Fulvestrant. These drugs were found not to interfere with the **cobas** PIK3CA Test when the drug was added to samples during the sample preparation procedure.

Robustness

Robustness of the **cobas** PIK3CA Test was determined using one PIK3CA H1047R mutant FFPET sample with ~11.0% mutation. The sample was sectioned into 100 individual 5-µm sections for analysis. DNA was extracted from each section using the **cobas**® DNA Sample Preparation Kit. A single replicate of the extracted DNA was tested for each of the 100 sections for the sample. 100% of the replicates were reported as "Mutation Detected" by the **cobas** PIK3CA Test for the H1047X mutation, yielding a false-negative rate of 0%.

Repeatability

Repeatability of the **cobas** PIK3CA Test was assessed using ten FFPET samples, including: 2 wild-type samples and 8 mutant samples each with one E542K, N345K, E545K, C420R, G1049R, Q546K, R88Q, or H1047R mutation. These samples were tested in duplicate by two operators, using two different reagent lots and two **cobas z** 480 analyzers over 8 days for each sample. The **cobas** PIK3CA Test had a correct call rate of 99.7% (319/320).

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Document Revision Information	
Doc Rev. 3.0 06/2020	Removed the DNA Sample Preparation kit information regarding reagent listings, reagent composition information, and related procedural steps and notes. Added reference to the **cobas**® DNA Sample Preparation Kit Instructions for Use at the beginning and in the **Procedural Limitations** section. Updated System and Operator Manual references throughout to "**cobas**® 4800 System – Operator's Manual or **cobas**® 4800 System – User Assistance". Made corrections to typos and updated for consistency and standardization of language throughout. Updated International Air Transport Association reference. Updated the harmonized symbol page. Updated trademarks and patents section. Added Roche web address www.roche.com. Please contact your local Roche Representative if you have any questions.

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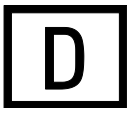

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06/2020 06671381001-04
Doc Rev. 3.0

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Biological risks

Catalogue number


Consult instructions for use

Contents of kit

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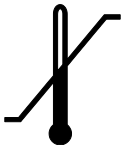
Lower Limit of Assigned Range


Manufacturer


Store in the dark

Contains sufficient for <*n*> tests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Test Definition File

Upper Limit of Assigned Range


Use-by date

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