Rx Only

cobas® PIK3CA Mutation Test

For in vitro diagnostic use

IVD

cobas® PIK3CA Mutation Test	24 Tests	P/N: 07003986190

For FFPET samples, refer to the **cobas®** DNA Sample Preparation Kit (M/N 05985536190) for sample preparation.

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Intended use

The **cobas**® PIK3CA Mutation Test is a real-time PCR test for the qualitative detection and identification of 17 mutations in exons 2, 5, 8, 10 and 21 in the gene encoding the catalytic subunit of phosphoinositide 3-kinase (PIK3CA) in DNA isolated from formalin-fixed paraffin-embedded tumor tissue (FFPET). The **cobas**® PIK3CA Mutation Test is a real-time PCR test for use on the **cobas**® 4800 System intended to be used to identify patients with metastatic breast cancer whose tumors harbor these mutations.

Summary and explanation of the test

Background

The phosphoinositide-3-kinase (PI3K) signaling pathway is a key regulator of many features of normal cell behavior, including growth, survival, motility, and proliferation.[1-3] Activation and dysregulation of the PI3K pathway have been implicated in a wide range of human cancers.[4] In cancer, an important mechanism of pathway activation occurs via receptors known as receptor tyrosine kinases (RTK), such as human epidermal growth factor receptor 2 (HER-2) or the epidermal growth factor receptor (EGFR). PI3K, Akt and mTOR comprise 3 major junctions in the PI3K/Akt/mTOR signaling pathway.[5] Activation of the PI3K pathway leads to conversion of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3), an important secondary messenger which stimulates a broad range of downstream effector pathways including Akt and mTOR. Activation of the PI3K/Akt/mTOR pathway leads to many cellular processes including cell growth, proliferation and survival.[6]

PIK3CA is one of the most highly mutated oncogenes identified in human cancers. Additionally, most of the reported mutations occur in a few hotspots in the protein (codons 1047 [40%], 545 [25%], and 542 [13%]).[7] Most of the mutations occur primarily in exons 10 and 21 of PIK3CA. However, activating mutations have also been found in exons 2, 5, and 8. In the *COSMIC* database, PIK3CA has been shown to be mutated 12% of the time across all tumor types. In breast cancer (BC), PIK3CA mutations were found in 26% of the tumors tested. PIK3CA mutations have been shown to be more common in hormone receptor-positive (~40%) and in HER-2 positive (~25%) BC.[8,9]

In women, BC is the most frequently diagnosed cancer and the leading cause of cancer death. In 2017, BC accounted for 25% of the total cancer cases and 15% of the cancer deaths in women worldwide.[10] About half the BC cases and 60% of the deaths are estimated to occur in economically developed countries. In general, incidence rates are high in Western and Northern Europe, Australia/New Zealand, and North America. The factors that contribute to the international variation in incidence rates largely stem from differences in reproductive and hormonal factors and the availability of early detection services.[11] Newer therapies are needed to combat the high incidence of BC in general and the large number of cancer deaths due to BC. Knowing the mutation status of PIK3CA is important in utilizing therapies that target the activity of the PIK3CA pathway.

The mutations detected by the **cobas**® PIK3CA Mutation Test (**cobas** PIK3CA Test) are listed in Table 1**.**

Table 1: Mutations detected by the cobas® PIK3CA Test

Current PIK3CA Exon Number	Former PIK3CA Exon Number*	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	HGVS** Protein Nomenclature	HGVS** Nucleotide Nomenclature	COSMIC ID[12]
2	1	R88Q	263G>A	NM_006218.2:p.(Arg88Gln)	NM_006218.2:c.263G>A	746
5	4	N345K	1035T>A	NM_006218.2:p.(Asn345Lys)	NM_006218.2:c.1035T>A	754
8	7	C420R	1258T>C	NM_006218.2:p.(Cys420Arg)	NM_006218.2:c.1258T>C	757
10	9	E542K	1624G>A	NM_006218.2:p.(Glu542Lys)	NM_006218.2:c.1624G>A	760
10	9	E545A	1634A>C	NM_006218.2:p.(Glu545Ala)	NM_006218.2:c.1634A>C	12458
10	9	E545D	1635G>T	NM_006218.2:p.(Glu545Asp)	NM_006218.2:c.1635G>T	765
10	9	E545G	1634A>G	NM_006218.2:p.(Glu545Gly)	NM_006218.2:c.1634A>G	764
10	9	E545K	1633G>A	NM_006218.2:p.(Glu545Lys)	NM_006218.2:c.1633G>A	763
10	9	Q546E	1636C>G	NM_006218.2:p.(Gln546Glu)	NM_006218.2:c.1636C>G	6147
10	9	Q546K	1636C>A	NM_006218.2:p.(Gln546Lys)	NM_006218.2:c.1636C>A	766
10	9	Q546L	1637A>T	NM_006218.2:p.(Glu546Leu)	NM_006218.2:c.1637A>T	25041
10	9	Q546R	1637A>G	NM_006218.2:p.(Gln546Arg)	NM_006218.2:c.1637A>G	12459
21	20	H1047L	3140A>T	NM_006218.2:p.(His1047Leu)	NM_006218.2:c.3140A>T	776
21	20	H1047R	3140A>G	NM_006218.2:p.(His1047Glu)	NM_006218.2:c.3140A>G	775
21	20	H1047Y	3139C>T	NM_006218.2:p.(His1047Tyr)	NM_006218.2:c.3139C>T	774
21	20	G1049R	3145G>C	NM_006218.2:p.(Gly1049Glu)	NM_006218.2:c.3145G>C	12597
21	20	M1043I	3129G>T	NM_006218.2:p.(Met1043Ile)	NM_006218.2:c.3129G>A	773

* Former PIK3CA exon numbering excluded the first untranslated exon

** HGVS – Human Genome Variation Society

Principles of the procedure

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 2, N345K in exon 5, C420R in exon 8, E542K, E545X (E545A, E545D*, E545G, and E545K), Q546X (Q546E, Q546K, Q546L, and Q546R) in exon 10, and M1043I**, H1047X (H1047L, H1047R, and H1047Y), and G1049R in exon 21. 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.

* 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.

Reference sequences

Please refer to the following source for the reference sequence for PIK3CA.[13]

PIK3CA: LRG_310t1

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 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 2, 5, 8, 10, and 21. An additional primer pair targets a conserved 167 base pair region in exon 4 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.

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, a 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 PIK3CA sequences 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).[14] 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 and materials

All unopened reagents and controls shall be stored as recommended in the Reagent storage and handling requirements table.

Reagents provided for the cobas® PIK3CA Mutation Test, 24 Tests (P/N: 07003986190)

Reagents	Reagent Ingredients	Quantity per Kit	Safety Symbol and Warning
PIK3CA MMX-1 (**PIK3CA Master Mix 1; Cap with White Button**) (P/N 07003897001)	Tris buffer, potassium chloride, glycerol, EDTA, non-ionic detergent, dimethyl sulfoxide, 0.09% sodium azide, dNTPs, DNA polymerase, AmpErase (uracil-N-glycosylase) enzyme (microbial), Aptamer, PIK3CA primers, fluorescent labeled PIK3CA probes	2 x 0.48 mL	N/A
PIK3CA MMX-2 (**PIK3CA Master Mix 2; Cap with Gold Button**) (P/N 07003927001)	Tris buffer, potassium chloride, glycerol, EDTA, non-ionic detergent, dimethyl sulfoxide, 0.09% sodium azide, dNTPs, DNA polymerase, AmpErase (uracil-N-glycosylase) enzyme (microbial), Aptamer, PIK3CA primers, fluorescent labeled PIK3CA probes	2 x 0.48 mL	N/A
PIK3CA MMX-3 (**PIK3CA Master Mix 3; Cap with Teal Button**) (P/N 07003943001)	Tris buffer, potassium chloride, glycerol, EDTA, non-ionic detergent, dimethyl sulfoxide, 0.09% sodium azide, dNTPs, DNA polymerase, AmpErase (uracil-N-glycosylase) enzyme (microbial), Aptamer, PIK3CA primers, fluorescent labeled PIK3CA probes	2 x 0.48 mL	N/A
MGAC (**Magnesium acetate; Cap with Yellow Button**) (P/N 05854326001)	Magnesium acetate, 0.09% Sodium azide	6 x 0.20 mL	N/A
PIK3CA MC (**PIK3CA Mutant Control; Cap with Red Button**) (P/N 07003960001)	Tris buffer, EDTA, Poly-rA RNA (synthetic), 0.05% sodium azide, Plasmid DNA containing PIK3CA exon 2, 5, 8, 10, and 21 sequences, PIK3CA wild-type DNA	6 x 0.10 mL	N/A
DNA SD (**DNA Specimen Diluent**) (P/N 05854474001)	Tris-HCl buffer, 0.09% Sodium azide	2 x 3.5 mL	N/A

Reagent storage and handling requirements

Reagent	Storage Temperature	Storage Time
cobas® PIK3CA Mutation Test*	2°C to 8°C	Once opened, stable for 4 uses over 90 days or until the expiration date indicated, whichever comes first.

***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. Working MMX must be stored at 2°C to 8°C in the dark. The prepared samples and controls must be added within 1 hour of preparation of the working MMX. Amplification must be started within 1 hour from the time that the processed samples and controls are added to the working MMX.

Additional materials required

Materials	P/N
Bleach	Any vendor
70% Ethanol	Any vendor
cobas® 4800 System Microwell Plate (AD-plate) and sealing film	Roche 05232724001
cobas® 4800 System sealing film applicator (supplied with the installation of the **cobas**® 4800 System)	Roche 04900383001
Adjustable pipettors* (Capable of pipetting 5-1000 µL)	Any vendor
Aerosol barrier or positive displacement DNase-free tips	Any vendor
Bench top microcentrifuge* (capable of 20,000 x g)	Eppendorf 5430 or 5430R or equivalent
Locking-lid microcentrifuge tubes (1.5mL sterile, RNase/DNase free, PCR grade)	Any vendor
Microcentrifuge tube racks	Any vendor
Spectrophotometer for measuring DNA concentration*	Any vendor
Vortex mixer*	Any vendor
Disposable gloves, powder-free	Any vendor

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

For more information regarding the materials sold separately, contact your local Roche representative.

Instrumentation and software required but not provided

Required Instrumentation and Software, Not Provided	P/N
cobas z 480 analyzer and Control Unit	05200881001
cobas® 4800 System Application Software (Core) version 2.2 or higher	07565500001
cobas® PIK3CA P1 Analysis Package Software version 1.0 or higher	08249628001

For more information regarding the materials sold separately, contact your local Roche representative.

Precautions and handling requirements

Warnings and precautions

As with any test procedure, good laboratory practice is essential to the proper performance of this assay.

- For in vitro diagnostic use only.
- Safety Data Sheets (SDS) are available upon request from your local Roche office.
- This test is for use with FFPET samples. Samples should be handled as if infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories[15] and in the CLSI Document M29-A4.[16]
- The use of sterile disposable pipettes and DNase-free pipettor tips is recommended.

Good laboratory practice

- Do not pipette by mouth.
- Do not eat, drink or smoke in laboratory work areas.
- Wash hands thoroughly after handling samples and kit reagents.
- 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.
- Thoroughly clean and disinfect all laboratory work surfaces with a freshly prepared solution of 0.5% sodium hypochlorite in distilled or deionized water (dilute household bleach 1:10). Follow by wiping the surface with 70% ethanol.

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.*

Contamination

- Gloves must be worn and must be changed between handling samples and **cobas** PIK3CA Test reagents to prevent contamination. Avoid contaminating gloves when handling samples.
- Gloves must be changed frequently to reduce the potential for contamination.
- Gloves must be changed before leaving DNA isolation areas or if contact with solutions or a sample is suspected.
- Avoid microbial contamination of reagents.
- The amplification and detection work area should be thoroughly cleaned before working MMX preparation. Supplies and equipment should be dedicated to each activity and not used for other activities or moved between areas. For example, pipettors and supplies used for DNA isolation must not be used to prepare reagents for Amplification and Detection.
- It is highly recommended that workflow in the laboratory proceed in a uni-directional manner, completing one activity before proceeding to the next activity. For example, DNA isolation should be completed before starting amplification and detection. DNA isolation should be performed in an area separate from amplification and detection. To avoid contamination of the working master mix with DNA samples, the amplification and detection work area should be thoroughly cleaned before working master mix preparation.

Integrity

- Do not use kits after their expiration dates.
- Do not pool reagents from different kits or lots.
- Do not combine reagent vials from different kit lots.
- Do not use disposable items beyond their expiration date.
- All disposable items are for one time use. Do not reuse.
- All equipment should be properly maintained according to the manufacturer's instructions.

Disposal

1. **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.
2. Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

Spillage and cleaning

- If spills occur on the **cobas**® 4800 instrument, follow the instructions in the **cobas**® 4800 System - User Assistance to clean.
- 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 - User Assistance.
- For additional warnings, precautions and procedures to reduce the risk of contamination for the **cobas z** 480 analyzer, consult the **cobas**® 4800 System - User Assistance.

Sample collection, transport, and storage

Note: *Handle all specimens as if they are capable of transmitting infectious agents.*

Sample collection

BC FFPET specimens have been validated for use with the **cobas** PIK3CA Test.

Sample transport, storage, and stability

BC FFPET specimens can be transported at 15°C to 30°C. Transportation of BC FFPET specimens must comply with country, federal, state, and local regulations for the transport of etiologic agents.[17]

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

BC FFPET samples are stable for either:

FFPET Sample Type	FFPET Block	5 µm FFPET Section
FFPET Sample Storage Temperature	15°C to 30°C	15°C to 30°C
Storage Time	Up to 12 months	Up to 60 days

Processed sample storage and stability

Processed samples (extracted DNA) are stable for one of the following:

Extracted DNA Storage Temperature	-15°C to -25°C	2°C to 8°C	15°C to 30°C
Storage Time	Up to 3 freeze thaws over 60 days	Up to 14 days	Up to 1 day

Extracted DNA should be used 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.

Test procedure

Running the test

Figure 1: cobas PIK3CA Test workflow with cobas® DNA Sample Preparation Kit

#	Workflow Step
1	Start the system
2	Perform instrument maintenance
3	Remove samples and reagents from storage
4	Deparaffinize samples
5	Perform DNA isolation
6	Elute DNA
7	Create work order and print plate layout
8	Prepare amplification reagents
9	Load AD-plate with amplification reagents
10	Load AD-plate with sample
11	Seal AD-plate
12	Load AD-plate on the **cobas z** 480 analyzer
13	Start the run
14	Review results
15	With LIS: send results to LIS
16	Unload analyzer

Instructions for use

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

Note: *Refer to the **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 AD plate. When running more than 24 samples, multiple **cobas** PIK3CA Test kits 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.

Full process controls

This test requires a full-process negative control (***NEG***). For each run, process a ***NEG*** concurrently with the sample(s) beginning with the DNA isolation procedure.

DNA isolation

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

Macro-dissection

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

DNA quantitation

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

Note: *Store DNA stock according to instructions in* ***Sample transport, storage, and stability*** *section.*

1. Mix each DNA stock by vortexing for 5 seconds.
2. Quantify DNA using a spectrophotometer according to the manufacturer's protocol. Use DNA Elution Buffer **(DNA EB)** as the blank for the instrument. An average of two consistent readings is necessary. The two measurements should be within ± 10% of each other when the DNA concentration readings are ≥ 20.0 ng/µL. For DNA concentration readings< 20.0 ng/µL, the two measurements should be within ± 2 ng/µL. If the two measurements are not within ± 10% of each other when the DNA concentration readings are ≥ 20.0 ng/µL or within ± 2 ng/µL when the DNA concentration readings are < 20.0 ng/µL, an additional 2 readings must be taken until the requirements are met. The average of these two new measurements should then be calculated.

Note: *The DNA stock from the processed* ***NEG*** *does not need to be measured.*

3. The DNA stock concentration from the samples must be ≥ 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 DNA Tissue Lysis Buffer (****DNA TLB)*** *+ Proteinase K (****PK****), and perform DNA isolation and quantitation as described above. For unmounted samples, combine two sections into one tube and perform deparaffinization. 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, request another FFPET sample section from the referring clinical site.*

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 – User Assistance for detailed instruction for the **cobas z** 480 analyzer set up.

Test order set-up

For detailed instructions on the **cobas** PIK3CA Test workflow steps, refer to the **cobas**® 4800 System – User Assistance.

Generate a plate map with the position of all the samples and controls in the run. The Mutant Control (MC) is loaded into positions A01 – A03 on the plate. The ***NEG*** is loaded into positions B01 – B03 on the plate. Diluted samples are then added in sets of 3 columns, starting from C01 – C03 through H10 – H12, as shown in Table 2 below.

Table 2: Plate layout for the cobas PIK3CA Test

Row / Column	01	02	03	04	05	06	07	08	09	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: MC= Mutant Control, NEG = Negative 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 response.*

Note: *Working Master Mix 1 must be loaded into column 01, 04, 07, and 10 on the plate. Working Master Mix 2 must be loaded into column 02, 05, 08, and 11 on the plate. Working Master Mix 3 must be loaded into column 03, 06, 09, and 12 on the plate.*

Note: *Up to 30 samples can be loaded onto a single plate. If more than one reagent kit is required to process all of the samples on the plate, then the kits must all be from the same lot.*

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 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).*

1. 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]

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

   µL of **DNA SD** = 90 µL – µL of DNA stock

Example:

DNA stock concentration = 6.5 ng/µL

1. µL of DNA stock = (90 µL x 2 ng/µL) ÷ 6.5 ng/µL = 27.7 µL
2. 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 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).*

1. At DNA stock concentrations > 36 ng/µL, use the following formula to calculate the amount of **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.
2. 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

1. Vol. of **DNA SD** required in µL = [(5 µL x 100 ng/µL) / 2 ng/µL] – 5 µL = 245 µL
2. Use the calculated volume of **DNA SD** to dilute 5 µL of DNA stock.

Sample dilution

1. Prepare the appropriate number of 1.5 mL locking-lid microcentrifuge tubes for DNA Dilutions by labeling them with the proper sample identification.
2. 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 locking-lid microcentrifuge tube labeled as **NEG**.
3. Vortex each DNA stock and the ***NEG*** for 5 to 10 seconds.
4. 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 ***NEG*** (extracted eluate) into the **NEG** tube.
5. Cap the tubes and vortex each for 5 to 10 seconds.
6. Change gloves.

Reaction set-up

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** reagents 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 MMXs, one containing **PIK3CA MMX-1**, one containing **PIK3CA MMX-2**, and the other containing **PIK3CA MMX-3** in separate 1.5 mL locking-lid microcentrifuge tubes.

1. 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

2. 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 3 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 3: Volumes of reagents needed for working MMX-1, working MMX-2 and working MMX-3 based upon number of samples* tested

Reagent	Volume	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

3. Remove the appropriate number of **PIK3CA MMX-1** or **PIK3CA MMX-2** or **PIK3CA MMX-3,** and **MGAC** vials from 2°C to 8°C storage. Vortex each reagent for 5 seconds and collect the 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.
4. Add the calculated volume of **PIK3CA MMX-1** or **PIK3CA MMX-2** or **PIK3CA MMX-3** to their respective working MMX tube.
5. Add the calculated volume of **MGAC** to the working MMX tubes.
6. 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 **cobas**® 4800 System AD-plate and Sealing film.*

Preparation of plate

1. Pipette 25 µL of working MMX into each reaction well of the AD-plate that is needed for the run. Do not allow the pipettor tip to touch the plate outside the well.
    - Add working MMX-1 (containing **PIK3CA MMX-1**) to the AD-plate wells in columns 01, 04, 07, and 10, as needed.
    - Add working MMX-2 (containing **PIK3CA MMX-2**) to the AD-plate wells in columns 02, 05, 08, and 11, as needed.
    - Add working MMX-3 (containing **PIK3CA MMX-3**) to the AD-plate wells in columns 03, 06, 09, and 12, as needed.
2. Pipette 25 µL of **PIK3CA MC** into wells **A01, A02,** and **A03** of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.
3. Using a new pipettor tip, pipette 25 µL of ***NEG*** into wells **B01, B02,** and **B03** 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 **PIK3CA MC** in wells **A01**, **A02** and **A03**, and **NEG** in wells **B01**, **B02**, and **B03** or the run will be invalidated by the **cobas z** 480 analyzer.*

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

4. Using new pipettor tips for each diluted sample DNA, add 25 µL of the first sample DNA to wells **C01**, **C02**, and **C03** of the AD-plate, using a new tip for the addition of the sample DNA to each well; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for the diluted DNA from each sample and follow the template in Table 2 until all samples' DNA Dilutions are loaded onto the AD-plate. Ensure that all liquid is collected at the bottom of the wells.
5. 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.
6. 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 – User Assistance for detailed instructions on the PIK3CA workflow steps. When the "Select test" pop-up window appears, select "PCR Only" workflow type, then check "PIK3CA P1" and click the "OK" button.

Results

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 4.

Table 4: Result interpretation of cobas PIK3CA Test

Test Result	Mutation Result	Interpretation
Mutation Detected	R88Q	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	N345K	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	C420R	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	E542K	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	E545X (E545A, E545D*, E545G, or E545K)	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	Q546X (Q546E, Q546K, Q546L, or Q546R)	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	M1043I**	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	H1047X (H1047L, H1047R, or H1047Y)	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	G1049R	Mutation detected in specified targeted PIK3CA region.
Mutation Detected	(More than one mutation may be present)	Mutation detected in specified targeted PIK3CA region.
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.
Failed	N/A	Failed run due to hardware or software failure. Contact your local Roche office for technical assistance.

* 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.

Note: *A “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.*

Retesting of samples with invalid results

1. Repeat dilution of the invalid sample DNA stock starting from the “Dilution calculation of sample DNA stock” and “Sample Dilution” procedures in the **Amplification and detection** section.
2. After performing the DNA stock dilution to 2 ng/µL as described in the “Sample dilution”, continue with “preparation of working MMX (MMX-1, MMX-2 and MMX-3)” 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-µm FFPET tumor section.*

Quality control and validity of results

One set of **cobas** PIK3CA Test **PIK3CA MC** (wells **A01, A02** and **A03**) and ***NEG*** (wells **B01, B02** and **B03)** 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** and ***NEG*** are valid. If a **PIK3CA MC** or ***NEG*** is 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.

Mutant control

The **PIK3CA MC** result must be 'Valid'. If the **PIK3CA MC** results are consistently invalid, contact your local Roche office for technical assistance.

Negative control

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

Procedural limitations

1. Test only the indicated specimen types. The **cobas** PIK3CA Test was verified using BC FFPET specimens.
2. The **cobas** PIK3CA Test has been validated using only 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 Instruction For Use, and in the **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 this Instruction 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.
9. Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to another, users perform method correlation studies in their laboratory to qualify technology differences.
10. The presence of PCR inhibitors may cause false negative or invalid results.
11. Though rare, mutations within the genomic DNA regions of the PIK3CA gene covered by the primers or probes used in **cobas** PIK3CA Test may lead to an incorrect result.

12. Though rare, the **cobas** PIK3CA Test may show some limited cross-reactivity (results of "Mutation Detected") for mutations flanking the targeted mutations in exons 10 and 21 (e.g., at high mutant percentages E545K may return a mutation result of E545X;Q546X or H1047X may return a mutation result of H1047X;G1049R).

13. The **cobas** PIK3CA Test was validated for use with 50 ng of DNA per reaction well. DNA input amounts lower than 50 ng per reaction well are not recommended.

14. The **cobas** PIK3CA Test is a qualitative test. The test is not for quantitative measurements of percent mutation.

15. BC FFPET specimens containing degraded DNA may affect the ability of the test to detect the PIK3CA mutations.

16. Samples with results reported as “No Mutation Detected” may harbor PIK3CA mutations not detected by the assay.

17. 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).

Non-clinical performance evaluation

Note: *The study descriptions below include cumulative data performed with the* ***cobas*** *PIK3CA Test.*

For the non-clinical studies described below, percentage of tumor was assessed by pathology review. Bi-directional Sanger sequencing and next generation sequencing (NGS) were used to select the specimens for testing. Percentage of mutation of BC FFPET specimen was determined using a NGS method.

Key performance characteristics

Analytical sensitivity – limit of blank (LoB)

To assess performance of the **cobas** PIK3CA Test and ensure that wild-type (WT) samples do not generate an analytical signal that might indicate a low concentration of mutation; BC FFPET PIK3CA WT samples were evaluated. The LoB was determined using the non-parametric option as prescribed in CLSI guideline EP17-A2[18] for PIK3CA WT samples. The LoB was determined to be zero for all mutations.

Limit of detection using FFPET sample blends

DNA isolated from 33 BC FFPET samples with PIK3CA mutations were blended with DNA isolated from 25 PIK3CA WT BC 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 an NGS method. Dilutions of each sample DNA blend were prepared and a total of 21 replicates of each mutation level were run using three **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 5.

Table 5: Limit of detection for the cobas PIK3CA Test using FFPET sample DNA blends

PIK3CA Exon	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	COSMIC ID[12]	Sample	Percent Mutation in the Panel Member to achieve ≥ 95% "Mutation Detected" Rate with 50 ng DNA input per reaction well (N = 21 replicates)
2	R88Q	263 G>A	746	Sample 1	2.2%
2	R88Q	263 G>A	746	Sample 2	1.3%
2	R88Q	263 G>A	746	Sample 3	1.1%
5	N345K	1035 T>A	754	Sample 4	2.2%
5	N345K	1035 T>A	754	Sample 5	1.9%
5	N345K	1035 T>A	754	Sample 6	1.3%
8	C420R	1258 T>C	757	Sample 7	1.7%
8	C420R	1258 T>C	757	Sample 8	1.9%
8	C420R	1258 T>C	757	Sample 9	1.6%
10	E542K	1624 G>A	760	Sample 10	1.1%
10	E542K	1624 G>A	760	Sample 11	1.2%
10	E542K	1624 G>A	760	Sample 12	1.1%
10	E545A	1634 A>C	12458	Sample 13	2.8%
10	E545A	1634 A>C	12458	Sample 14	0.9%
10	E545A	1634 A>C	12458	Sample 15	1.6%
10	E545G	1634 A>G	764	Sample 16	1.8%
10	E545G	1634 A>G	764	Sample 17	1.2%
10	E545G	1634 A>G	764	Sample 18	1.6%
10	E545K	1633 G>A	763	Sample 19	3.3%
10	E545K	1633 G>A	763	Sample 20	1.5%
10	E545K	1633 G>A	763	Sample 21	1.8%
10	Q546E	1636 C>G	6147	Sample 22	3.5%
10	Q546E	1636 C>G	6147	Sample 23	1.6%
10	Q546E	1636 C>G	6147	Sample 24	2.5%
10	Q546K	1636 C>A	766	Sample 25	3.4%
10	Q546K	1636 C>A	766	Sample 26	2.3%
10	Q546K	1636 C>A	766	Sample 27	2.7%
10	Q546R	1637 A>G	12459	Sample 28	1.5%
10	Q546R	1637 A>G	12459	Sample 29	3.2%
10	Q546R	1637 A>G	12459	Sample 30	1.3%
21	H1047L	3140 A>T	776	Sample 31	2.8%
21	H1047L	3140 A>T	776	Sample 32	1.8%
21	H1047L	3140 A>T	776	Sample 33	3.3%
21	H1047R	3140 A>G	775	Sample 34	2.8%
21	H1047R	3140 A>G	775	Sample 35	1.5%
21	H1047R	3140 A>G	775	Sample 36	1.0%
21	H1047Y	3139 C>T	774	Sample 37	3.5%
21	H1047Y	3139 C>T	774	Sample 38	2.2%
21	H1047Y	3139 C>T	774	Sample 39	3.4%
21	G1049R	3145 G>C	12597	Sample 40	1.0%
21	G1049R	3145 G>C	12597	Sample 41	0.7%
21	G1049R	3145 G>C	12597	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 6, a DNA plasmid construct was blended with WT 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% upper confidence limit for each plasmid blend are shown in Table 6.

Table 6: Mutations detected by the cobas PIK3CA Test using mutant plasmid DNA blends

PIK3CA Exon	PIK3CA Mutation	PIK3CA Nucleic Acid Sequence	COSMIC ID[12]	Actual Percent Mutation	Binomial 95% lower confidence limit (N ≥ 20)	Binomial 95% upper confidence limit (N ≥ 20)
10	E545D	1635 G>T	765	1.2%	62%	97%
10	Q546L	1637 A>T	25041	2.1%	83%	100%
21	M1043I	3129 G>T	773	2.6%	83%	100%

Repeatability

Repeatability of the **cobas** PIK3CA Test was assessed using ten FFPET samples, including: two WT samples and eight 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 eight days. A total of 32 replicates of each sample were evaluated. The **cobas** PIK3CA Test had a correct call rate of 99.7% (319/320).

Correlation to reference method

Comparison testing of 206 BC FFPET samples using each of two lots of the **cobas** PIK3CA Test and Sanger sequencing (Sanger) was performed to determine positive, negative and overall percent agreement between methods. Discordant results between the **cobas** PIK3CA Test and Sanger were tested using NGS to resolve discordance.

cobas PIK3CA Test and Sanger results

The comparison of the 205 valid results for Sanger and the **cobas** PIK3CA Test is shown in Table 7.

Table 7: Agreement analysis of cobas PIK3CA Test vs. Sanger

	Sanger, MD	Sanger, NMD	**Total**
cobas PIK3CA Test, MD	95*	7	102
cobas PIK3CA Test, NMD	0	103	103
cobas PIK3CA Test, 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 for Lot 1 and three samples for Lot 2 were MD by both Sanger sequencing and the **cobas** PIK3CA Test, but Sanger sequencing detected the first mutation and missed the second mutation (see Table 9).

The comparison between the **cobas** PIK3CA Test and Sanger evaluated nine targets for each sample. A total of 1845 calls were made based on the results of the 205 valid samples. Table 8 shows the comparison of the **cobas** PIK3CA Test and Sanger on a per call basis for Lot 1.

Table 8: Per call comparison of the cobas® PIK3CA Test vs. Sanger for Lot 1

	Sanger, R88Q	Sanger, N345K	Sanger, C420R	Sanger, E542K	Sanger, E545X	Sanger, Q546X	Sanger, M1043I	Sanger, H1047X	Sanger, G1049R	Sanger, NMD	**Total**
cobas PIK3CA Test, R88Q	1	-	-	-	-	-	-	-	-	-	1
cobas PIK3CA Test, N345K	-	7	-	-	-	-	-	-	-	-	7
cobas PIK3CA Test, C420R	-	-	3	-	-	-	-	-	-	-	3
cobas PIK3CA Test, E542K	-	-	-	14	-	-	-	-	-	2	16
cobas PIK3CA Test, E545X	-	-	-	-	17	-	-	-	-	2	19
cobas PIK3CA Test, Q546X	-	-	-	-	-	8	-	-	-	1*	9
cobas PIK3CA Test, M1043I	-	-	-	-	-	-	-	-	-	-	0
cobas PIK3CA Test, H1047X	-	-	-	-	-	-	-	42	-	7*	49
cobas PIK3CA Test, G1049R	-	-	-	-	-	-	-	-	3	-	3
cobas PIK3CA Test, NMD	-	-	-	-	-	-	-	-	-	1738	1738
cobas PIK3CA Test, Invalid	-	-	-	-	-	-	-	-	-	9	9
Total	1	7	3	14	17	8	0	42	3	1759	1854

*Results for Lot 2 were similar to Lot 1, except there were a total of two less discordant results for Lot 2. See Sample 8 and Sample 9 in Table 9.

Discordant analysis by NGS

Seven sample mutation 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 NGS and are shown in Table 9. A revised agreement analysis was performed based on the NGS results. In this analysis, samples with NGS results that agreed with the **cobas** PIK3CA Test result were considered concordant.

Table 9: Discordant result resolution by NGS

Sample	Sanger	cobas PIK3CA Test, Lot 1	NGS Resolution, Lot 1**	cobas PIK3CA Test, Lot 2	NGS Resolution, Lot 2**
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, therefore no resolution testing was necessary.

**The NGS for resolution was performed only for the exon(s) with the discordant result.

Note: *The **cobas** PIK3CA Test results for Samples 8 - 12 detected the same PIK3CA mutations as Sanger, however additional mutations were detected and confirmed by NGS.*

After the **cobas** PIK3CA Test vs. Sanger discordant results were resolved by NGS, the overall concordance of the **cobas** PIK3CA Test with sequencing was 100% across all targeted mutations for each lot, as shown in Table 10.

Table 10: Agreement analysis of the cobas PIK3CA Test vs. Sanger with discordant resolution by NGS (Sanger+NGS)

	Sanger+NGS, MD	Sanger+NGS, NMD	**Total**
cobas PIK3CA Test, MD	102	0	102
cobas PIK3CA Test, NMD	0	103	103
cobas PIK3CA Test, 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 11 shows the comparison of the **cobas** PIK3CA Test and Sanger with discordant resolution by NGS on a per call basis for Lot 1.

Table 11: Per call comparison of the cobas PIK3CA Test vs. Sanger with discordant resolution by NGS (Sanger + NGS)

	Sanger+ NGS, R88Q	Sanger+ NGS, N345K	Sanger+ NGS, C420R	Sanger+ NGS, E542K	Sanger+ NGS, E545X	Sanger+ NGS, Q546X	Sanger+ NGS, M1043I	Sanger+ NGS, H1047X	Sanger+ NGS, G1049R	Sanger+ NGS, NMD	**Total**
cobas PIK3CA Test, R88Q	1	-	-	-	-	-	-	-	-	-	1
cobas PIK3CA Test, N345K	-	7	-	-	-	-	-	-	-	-	7
cobas PIK3CA Test, C420R	-	-	3	-	-	-	-	-	-	-	3
cobas PIK3CA Test, E542K	-	-	-	16	-	-	-	-	-	-	16
cobas PIK3CA Test, E545X	-	-	-	-	19	-	-	-	-	-	19
cobas PIK3CA Test, Q546X	-	-	-	-	-	9*	-	-	-	-	9
cobas PIK3CA Test, M1043I	-	-	-	-	-	-	-	-	-	-	0
cobas PIK3CA Test, H1047X	-	-	-	-	-	-	-	49*	-	-	49
cobas PIK3CA Test, G1049R	-	-	-	-	-	-	-	-	3	-	3
cobas PIK3CA Test, NMD	-	-	-	-	-	-	-	-	-	1738*	1738
cobas PIK3CA Test, Invalid	-	-	-	-	-	-	-	-	-	9	9
Total	1	7	3	16	19	9	0	49	3	1747	1854

*Results for Lot 2 were similar to Lot 1, except there were a total of two less mutations detected by the cobas PIK3CA Test and Sanger + NGS. See Sample 8 and Sample 9 in Table 9. Lot 2 results had two more NMD and correspondingly one less Q546X mutation and one less H1047X mutation.

Cross-reactivity

The following non-targeted mutations were tested using plasmids for cross-reactivity at an approximate 50% plasmid in genomic DNA input level: M1043I, M1043V, M1043T, G1049S, G1049A, E542V, E542Q, E545D, 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 WT and mutant PIK3CA sequences.

Evaluation of potentially interfering substances

Endogenous

Triglycerides (≤ 37mM, CLSI recommended high concentration[19]) and hemoglobin (≤ 2 mg/mL, CLSI recommended high concentration[19]) have been shown not to interfere with the **cobas** PIK3CA Test when the substance was added to samples during the sample preparation procedure. In addition, samples with up to 90% adipose 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.

Necrotic tissue

BC FFPET samples with necrotic tissue content up to 55% for PIK3CA mutant and 70% in WT samples have been shown not to interfere with the call results using the **cobas** PIK3CA Test.

Clinical performance evaluation

Clinical reproducibility study

A study was performed to assess the reproducibility of the **cobas** PIK3CA Test across 3 testing sites, 1 internal and 2 external (2 operators per site), 3 reagent lots, and 5 non-consecutive testing days, with a 21-member panel of DNA samples extracted from FFPET sections of breast cancer Wild Type (WT) and Mutant type (MT) tumor specimens obtained from commercial tissue repositories. This panel included mutations in exons 2, 5, 8, 10, and 21, confirmed by DNA sequencing. A total of 3,780 tests were performed on the 21 panel members in 90 valid runs; all test results were valid. There were "No Mutation Detected" results in 180 valid tests of WT panel members, producing 100% agreement. Agreements were 100% for 19 of the 20 MT panel members. For panel member Exon 10 E545 – LOD, agreement was 99.4% (179 of 180 test results were Mutation Detected). Results by overall agreement are presented in Table 12. The coefficient of variation (CV) was < 2% in all mutation panel members. For Internal Controls the CV was < 1.9%. The CV% was < 0.4% for between lots and < 1.9% for within-lot.

Table 12: Overall estimates of agreement by panel member for the reproducibility study

Panel Member	Number of Valid Tests	Agreement N	Agreement % (95% CI)[a]
Wild Type	180	180	100 (98.0, 100.0)
Exon 5 N345K - LOD	180	180	100 (98.0, 100.0)
Exon 10 E542K - LOD	180	180	100 (98.0, 100.0)
Exon 10 E545K - LOD	180	180	100 (98.0, 100.0)
Exon 21 H1047L - LOD	180	180	100 (98.0, 100.0)
Exon 21 G1049R - LOD	180	180	100 (98.0, 100.0)
Exon 5 N345K - 2X LOD	180	180	100 (98.0, 100.0)
Exon 10 E542K - 2X LOD	180	180	100 (98.0, 100.0)
Exon 10 E545K - 2X LOD	180	180	100 (98.0, 100.0)
Exon 21 H1047L - 2X LOD	180	180	100 (98.0, 100.0)
Exon 21 G1049R - 2X LOD	180	180	100 (98.0, 100.0)
Exon 2 R88Q - LOD	180	180	100 (98.0, 100.0)
Exon 8 C420R - LOD	180	180	100 (98.0, 100.0)
Exon 10 E545A - LOD	180	179	99.4 (96.9, 100.0)
Exon 10 Q546K - LOD	180	180	100 (98.0, 100.0)
Exon 21 H1047R - LOD	180	180	100 (98.0, 100.0)
Exon 2 R88Q - 2X LOD	180	180	100 (98.0, 100.0)
Exon 8 C420R - 2X LOD	180	180	100 (98.0, 100.0)
Exon 10 E545A - 2X LOD	180	180	100 (98.0, 100.0)
Exon 10 Q546K - 2X LOD	180	180	100 (98.0, 100.0)
Exon 21 H1047R - 2X LOD	180	180	100 (98.0, 100.0)

Note: *Results are included as agreement when a valid test of Mutant panel member has a result of 'Mutation Detected' or when a valid test of Wild Type panel member has a result of 'No Mutation Detected'.*

[a] 95% CI = 95% exact confidence interval.

In summary, the reproducibility of the **cobas** PIK3CA Test for identification of mutations in exons 2, 5, 8, 10, and 21 of the PIK3CA gene in DNA derived from formalin-fixed paraffin-embedded human breast cancer tissue was excellent and the agreement was > 99% for all mutations evaluated in this study.

Result flags

Explanation of result flags

The source of a flag is indicated in the flag code as outlined in Table 13.

Table 13: Flag source

Flag code starts with	Flag source	Example
M*	Multiple or other reasons	M6
R	Result interpretation	R500
Z*	Analyzer	Z1

*Refer to the **cobas**® 4800 System – User Assistance.

All the result flags of the system that are user relevant are listed in Table 14.

Table 14: List of result interpretation flags

Flag code	Description	Recommended action
R500–R511	Mutant Control could not be detected	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that the elbow determination algorithm encountered an error. This may occur in the event of an atypical or noisy fluorescence pattern.
R512–R523	Mutant Control could not be detected	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that a negative result occurred for the Mutant Control (i.e. Mutant Control DNA may have not been added to one or more wells).
R524–R535	Mutant Control is out of range	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that an observed elbow value for the Mutant Control was below the established threshold (i.e. elbow too low). This may occur in the event of DNA contamination.
R536-R547	Mutant Control is out of range	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that an observed elbow value for the Mutant Control was above the established threshold (i.e. elbow too high). This may occur in the event of: 1. Incorrect preparation of working Master Mix, 2. Pipetting error when adding working Master Mix into a reaction well of the AD-plate, or 3. Pipetting error when adding Mutant Control into a reaction well of the AD-plate.
R548-R559	Negative Control could not be detected	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate that the elbow determination algorithm encountered an error. This may occur in the event of an atypical or noisy fluorescence pattern.
R560-R571	Negative Control is out of range	Repeat the run. Refer to the **Test procedure** section. These flag codes indicate a positive result occurred for the Negative Control (i.e. a contamination event occurred).

Flag code	Description	Recommended action
R572–R583	No target could be detected	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate the elbow determination algorithm encountered an error. This may occur in the event of an atypical or noisy fluorescence pattern.
R584–R586, R588–R590, R592–R594, R596–R604	Result is out of range	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate either: 1. An atypically low elbow value was observed for the sample, or 2. An atypical relationship between the Mutant elbow value and the Internal Control elbow value was observed for the sample.
R587, R591, R595	Internal control is out of range	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate an atypically low Internal Control elbow value was observed for the sample. This may occur if the PCR mixture is significantly overloaded with concentrated genomic DNA.
R605–R610	Internal control could not be detected	Repeat the sample. Refer to the **Test procedure** section. These flag codes indicate that the Internal Control result for the sample was not valid. The absence of a valid Internal Control result is suggestive of: 1. Poor quality genomic DNA from the sample, 2. Inadequate sample processing, 3. The presence of PCR inhibitors in the sample, 4. Rare mutations within the regions of the genomic DNA covered by the Internal Control primers and/or probes, 5. Sample DNA may have not been added to one or more wells, or 6. Other factors.

Additional information

Key assay features

Sample type	Formalin-Fixed Paraffin-Embedded Tissue (FFPET)
Minimum amount of sample required	5-µm FFPET section
Analytical sensitivity	5% mutant sequence in 50 ng of DNA
Analytical specificity	100% agreement with sequencing
Genotypes detected	R88Q N345K C420R E542K E545X (E545A, E545D*, E545G, or E545K) Q546X (Q546E, Q546K, Q546L, or Q546R) M1043I** H1047X (H1047L, H1047R, or H1047Y) G1049R

* 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.

Symbols

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

Table 15: Symbols used in labeling for Roche PCR diagnostic products

Age/DOB	Age or Date of Birth
	Ancillary Software
Assigned Range [copies/mL]	Assigned Range (copies/mL)
Assigned Range [IU/mL]	Assigned Range (IU/mL)
EC REP	Authorized representative in the European Community
BARCODE	Barcode Data Sheet
LOT	Batch code
	Biological risks
REF	Catalogue number
Collect Date	Collect date
	Consult instructions for use
	Contains sufficient for <*n*> tests
CONTENT	Contents of kit
CONTROL	Control
	Date of manufacture
D	Distributed by
	Do not re-use
	Female
	For IVD performance evaluation only
GTIN	Global Trade Item Number
IVD	*In vitro* diagnostic medical device
LLR	Lower Limit of Assigned Range
	Male
	Manufacturer
CONTROL −	Negative control
NON STERILE	Non sterile
#	Patient number
?	Patient Name


Peel here

CONTROL + Positive control

QS copies/PCR QS copies per PCR reaction, use the QS copies per PCR reaction in calculation of the results.

QS IU/PCR QS IU per PCR reaction, use the QS International Units (IU) per PCR reaction in calculation of the results.

SN Serial number

Site Site

Procedure Standard Standard Procedure

STERILE EO Sterilized using ethylene oxide


Store in the dark


Temperature limit

Test Definition File

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


This way up

UDI Unique Device Identification

Procedure UltraSensitive Ultrasensitive Procedure

ULR Upper Limit of Assigned Range

Urine Fill Line Urine Fill Line

Rx Only US Only: Federal law restricts this device to sale by or on the order of a physician.


Use-by date


Device for near-patient testing


Device Not for Near Patient Testing


Device for self-testing


Device not for self-testing

Technical support

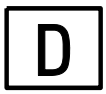
For technical support (assistance), please reach out to your local affiliate:
https://www.roche.com/about/business/roche_worldwide.htm

Manufacturer and distributors

Table 16: Manufacturer and distributors

Manufactured in the United States

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



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

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim, Germany

Trademarks and patents

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Document revision

Document Revision Information	
Doc Rev. 1.0 09/2020	First Publishing.