

cobas[®] **EGFR Mutation Test v2**

For in vitro diagnostic use



Kit	cobas [®] DNA Sample Preparation	24 Tests	P/N: 05985536190
	cobas [®] EGFR Mutation Test v2	24 Tests	P/N: 07248563190

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Intended Use

The **cobas**® EGFR Mutation Test v2 is a real-time PCR test for the qualitative detection of defined mutations of the epidermal growth factor receptor (EGFR) gene in DNA derived from formalin-fixed paraffin-embedded tumor tissue (FFPET) from non-small cell lung cancer (NSCLC) patients. The test is intended to aid in identifying patients with NSCLC whose tumors have defined EGFR mutations and for whom safety and efficacy of a drug have been established as follows:

Tarceva® (erlotinib)	Exon 19 deletions and L858R
Tagrisso® (osimertinib)	T790M

Drug safety and efficacy have not been established for the following EGFR mutations also detected by the **cobas**® EGFR Mutation Test v2:

Tarceva® (erlotinib)	G719X, exon 20 insertions, T790M, S768I and L861Q
Tagrisso® (osimertinib)	G719X, exon 19 deletions, L858R, exon 20 insertions, S768I, and L861Q

For manual sample preparation, FFPET specimens are processed using the **cobas**® DNA Sample Preparation Kit and the **cobas z** 480 analyzer is used for automated amplification and detection.

Summary and explanation of the test

Background

Activating mutations in the gene encoding EGFR occur primarily in NSCLC, and result in constitutive activation of the kinase activity of the EGFR protein, thereby contributing to the oncogenic process.¹ The prevalence of these mutations in unselected cases of NSCLC is approximately 10% - 30%.^{2,3} However, these mutations occur more frequently, but not exclusively, in non-smoking/light-smoking female patients of Asian ancestry with adenocarcinoma histologies.⁴

The most common EGFR mutations in NSCLC include a variety of deletions in exon 19 and the substitution mutation L858R in exon 21; these mutations collectively constitute approximately 85% of EGFR mutations observed in NSCLC.⁵ The **cobas**® EGFR Mutation Test v2 (**cobas**® EGFR Test) is a real-time PCR assay designed to detect G719X substitution mutations in exon 18, deletion mutations in exon 19, T790M and S768I substitution mutations and insertion mutations in exon 20, and L858R and L861Q substitution mutations in exon 21. The **cobas**® EGFR Test is used as a companion diagnostic test for Tarceva®, a compound that reversibly inhibits the kinase activity of EGFR, preventing autophosphorylation of tyrosine residues associated with the receptor and thereby inhibiting further downstream signaling that promotes cell survival and proliferation. Erlotinib binding affinity for EGFR exon 19 deletion or exon 21 L858R mutations is higher than its affinity for the wild-type receptor.⁶ Clinical trials have shown that patients with advanced NSCLC and with exon 19 deletion mutations or L858R substitution mutation in exon 21 that were treated with Tarceva® as first-line treatment, are likely to experience clinical benefit compared to patients treated with chemotherapy.^{3,7}

Tagrisso® (osimertinib), an irreversible inhibitor of both EGFR TKI-sensitizing and T790M resistance mutations in advanced NSCLC, inhibits the kinase activity of EGFR, which inhibits a cascade of intracellular downstream signaling events that promote cell proliferation, survival, and angiogenesis. Clinical trials have shown that patients with advanced non-squamous NSCLC with an EGFR TKI-sensitizing mutation and have progressed following therapy with a first generation EGFR TKI and who have developed a T790M resistance mutation in exon 20 that were treated with Tagrisso® (osimertinib) are likely to experience clinical benefit.

The mutations detected by the **cobas**[®] EGFR Test are listed in Table 1.

Table 1 The cobas® EGFR Test is designed to detect the following mutations

Exon	EGFR Mutation Group	EGFR Nucleic Acid Sequence	COSMIC ID ⁶
Exon 18	G719X	2156G>C	6239
		2155G>A	6252
		2155G>T	6253
Exon 19	Ex19Del	2240_2251del12	6210
		2239_2247del9	6218
		2238_2255del18	6220
		2235_2249del15	6223
		2236_2250del15	6225
		2239_2253del15	6254
		2239_2256del18	6255
		2237_2254del18	12367
		2240_2254del15	12369
		2240_2257del18	12370
		2239_2248TTAAGAGAAG>C	12382
		2239_2251>C	12383
		2237_2255>T	12384
		2235_2255>AAT	12385
		2237_2252>T	12386
		2239_2258>CA	12387
		2239_2256>CAA	12403
		2237_2253>TTGCT	12416
		2238_2252>GCA	12419
		2238_2248>GC	12422
		2237_2251del15	12678
		2236_2253del18	12728
		2235_2248>AATTC	13550
		2235_2252>AAT	13551
		2235_2251>AATTC	13552
		2253_2276del24	13556
2237_2257>TCT	18427		
2238_2252del15	23571		
2233_2247del15	26038		
Exon 20	S768I	2303G>T	6241
	T790M	2369C>T	6240
	Ex20Ins	2307_2308ins9GCCAGCGTG	12376
		2319_2320insCAC	12377
		2310_2311insGGT	12378
		2311_2312ins9GCGTGGACA	13428
2309_2310AC>CCAGCGTGGAT	13558		
Exon 21	L858R	2573T>G	6224
		2573_2574TG>GT	12429
	L861Q	2582T>A	6213

Principles of the procedure

The cobas® EGFR Test is based on two major processes: (1) manual specimen preparation to obtain DNA from FFPET; and (2) PCR amplification and detection of target DNA using complementary primer pairs and oligonucleotide probes labeled with fluorescent dyes. The cobas® EGFR Test is designed to detect the following mutations:

- Exon 18: G719X (G719A, G719C, and G719S)
- Exon 19: deletions and complex mutations
- Exon 20: S768I, T790M, and insertions
- Exon 21: L858R and L861Q

Mutation detection is achieved through PCR analysis with the cobas z 480 analyzer. A mutant control and negative control are included in each run to confirm the validity of the run.

Sample preparation

FFPET specimens are processed and genomic DNA isolated using the cobas® DNA Sample Preparation Kit, a manual specimen preparation based on nucleic acid binding to glass fibers. A deparaffinized 5- μ m section of an FFPET specimen is lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released genomic DNA from DNases. Subsequently, isopropanol is added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the genomic DNA is bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and then eluted with an aqueous solution. The amount of genomic DNA is spectrophotometrically determined and adjusted to a fixed concentration to be added to the amplification/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® EGFR Test.

PCR amplification

Target selection

The cobas® EGFR Test uses primers that define specific base-pair sequences for each of the targeted mutations. For the G719X substitution mutation in exon 18, sequences ranging from 104-106 base pairs are targeted; for the exon 19 deletion mutations, sequences ranging from 125 to 141 base pairs are targeted; for the S768I substitution mutation in exon 20, a 133 base pair sequence is targeted; for the T790M substitution mutation in exon 20, a 118 base pair sequence is targeted; for the exon 20 insertion mutations, sequences ranging from 125 to 143 base pairs are targeted; for the L858R substitution mutation in exon 21, a 138 base pair sequence is targeted; for the L861Q substitution mutation in exon 21, a 129 base pair sequence is targeted; for the internal control in exon 28, an 87 base pair sequence is targeted. Amplification occurs only in the regions of the EGFR gene between the primers; the entire EGFR gene is not amplified.

Target amplification

A derivative of *Thermus* species Z05-AS1 DNA polymerase is utilized for target amplification. First, the PCR mixture is heated to denature the genomic DNA and expose the primer target sequences. As the mixture cools, the upstream and downstream primers anneal to the target DNA sequences. The Z05 DNA polymerase, in the presence of divalent metal cation 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 EGFR 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® EGFR 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. Three different reporter dyes are used to label the mutations targeted by the test. Amplification of the seven targeted EGFR sequences are detected independently across three reactions by measuring fluorescence at the three characteristic wavelengths in dedicated optical channels.

Selective amplification

Selective amplification of target nucleic acid from the sample is achieved in the cobas® EGFR Test by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP).⁸ The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing thymidine. Deoxyuridine is not present in naturally occurring DNA but is always present in amplicon due to the use of dUTP in place of 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.

Materials and reagents

Materials and reagents provided

Kit/Cassettes	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning ^a
cobas® DNA Sample Preparation Kit 24 Tests (P/N: 05985536190)	DNA TLB (DNA Tissue Lysis Buffer) (P/N: 05517613001) Tris-HCl buffer Potassium chloride 0.04% EDTA 0.1% Triton X-100 0.09% Sodium azide	1 x 10 mL	 <p>Danger H302 + H332: Harmful if swallowed or if inhaled. H315: Causes skin irritation. H317: May cause an allergic skin reaction. H318: Causes serious eye damage. H334: May cause allergy or asthma symptoms or breathing difficulties if inhaled. H335: May cause respiratory irritation. P261: Avoid breathing dust/fume/gas/mist/vapours/spray. P280: Wear protective gloves/eye protection/face protection. P284 Wear respiratory protection. P305 + P351 + P338 + P310: IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/physician. P342 + P311: If experiencing respiratory symptoms: Call a POISON CENTER or doctor/physician. P362 + P364: Take off contaminated clothing and wash before reuse.</p>
	PK (Proteinase K) (P/N: 05860695102) Proteinase K, lyophilized	1 x 100 mg	
	DNA PBB (DNA Paraffin Binding Buffer) (P/N: 05517621001) Tris-HCl buffer 49.6% Guanidine hydrochloride 0.05% Urea 17.3% Triton X-100	1 x 10 mL	
	WB I (DNA Wash Buffer I) (P/N: 05517656001) Tris-HCl buffer 64% Guanidine hydrochloride	1 x 25 mL	
	WB II (DNA Wash Buffer II) (P/N: 05517664001) Tris-HCl buffer Sodium chloride	1 x 12.5 mL	
	DNA EB (DNA Elution Buffer) (P/N: 05517630001) Tris-HCl buffer 0.09% Sodium azide	1 x 6 mL	
	FT (Filter tubes with caps) (P/N: 05089506102)	1 x 25 pcs	
	CT (Collection Tubes) (P/N: 05880513001)	3 x 25 pcs	

Kit/Cassettes	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning ^a
cobas® EGFR Mutation Test v2 24 Tests (P/N: 07248563190)	EGFR MMX-1 (EGFR Master Mix 1) (P/N 06471366001) Tris buffer Potassium chloride Glycerol EDTA Tween 20 3.13% Dimethyl sulfoxide 0.09% Sodium azide < 0.10% dNTPs < 0.01% Z05-AS1 DNA polymerase (microbial) <0.01% AmpErase (uracil-N-glycosylase) enzyme (microbial) < 0.01% Aptamer < 0.01% Upstream and downstream EGFR primers < 0.01% Fluorescent labeled EGFR probes	2 x 0.48 mL	N/A
	EGFR MMX-2 (EGFR Master Mix 2) (P/N 06471382001) Tris buffer Potassium chloride Glycerol EDTA Tween 20 3.13% Dimethyl sulfoxide 0.09% Sodium azide <0.10% dNTPs < 0.01% Z05-AS1 DNA polymerase (microbial) < 0.01% AmpErase (uracil-N-glycosylase) enzyme (microbial) < 0.01% Aptamer < 0.01% Upstream and downstream EGFR primers < 0.01% Fluorescent labeled EGFR probes	2 x 0.48 mL	N/A
	EGFR MMX-3 v2 (EGFR Master Mix 3) (P/N 07248610001) Tris buffer Potassium chloride Glycerol EDTA Tween 20 3.13% Dimethyl sulfoxide 0.09% Sodium azide < 0.10% dNTPs < 0.01% Z05-AS1 DNA polymerase (microbial) < 0.01% AmpErase (uracil-N-glycosylase) enzyme (microbial) < 0.01% Aptamer < 0.01% Upstream and downstream EGFR primers < 0.01% Fluorescent labeled EGFR probes	2 x 0.48 mL	N/A

Kit/Cassettes	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning ^a
cobas® EGFR Mutation Test v2 24 Tests (P/N: 07248563190)	MGAC (Magnesium acetate) (P/N 05854326001) Magnesium acetate 0.09% Sodium azide	6 x 0.2 mL	N/A
	EGFR MC (EGFR Mutant Control) (P/N 06471455001) Tris buffer EDTA Poly-rA RNA (synthetic) 0.05% Sodium azide < 0.1% Plasmid DNA containing EGFR exon 18, 19, 20 and 21 sequences (microbial) < 0.1% EGFR wild-type DNA (cell culture)	6 x 0.1 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

Reagent	Storage Temperature	Storage Time
cobas® DNA Sample Preparation Kit*	15°C to 30°C	Once opened, stable up to 8 uses over 90 days or until the expiration date indicated, whichever comes first.
cobas® EGFR Mutation Test v2**	2°C to 8°C	Once opened, stable for 4 uses over 90 days or until the expiration date indicated, whichever comes first.

Note: With the exception of the PK reagent, do not freeze reagents.

* After addition of sterile, nuclease free water to **PK**, store unused reconstituted **PK** in 450 µL aliquots at -20°C. Once reconstituted, **PK** must be used within 90 days or until the expiration date, whichever comes first. After addition of absolute ethanol, store **WB I** and **WB II** at 15°C to 30°C. These working solutions are stable for 90 days or until the expiration date, whichever comes first.

** **EGFR MMX-1, EGFR MMX-2, EGFR MMX-3 v2**, and working MMX (prepared by the addition of **MGAC** to **EGFR MMX-1 or EGFR MMX-2 or EGFR MMX-3 v2**) 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
Xylene (ACS, > 98.5% xylenes)	Any vendor
Absolute ethanol (200 proof, for Molecular Biology)	Sigma E7023 or Fisher Scientific BP2818-500 or equivalent
Isopropanol (ACS, > 99.5%)	Sigma 190764 or Fisher Scientific A451-1 or equivalent
Sterile, nuclease-free water (for Molecular Biology)	Applied Biosystems (Ambion) AM9937 or GE Healthcare Hyclone™ SH3053801 or equivalent
Bleach	Any vendor
70% Ethanol	Any vendor
Sterile disposable, serological 5- and 25- mL pipettes	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
Pipet-Aid™*	Drummond 4-000-100 or equivalent
Bench top microcentrifuge* (capable of 20,000 x g)	Eppendorf 5430 or 5430R or equivalent
Two dry heat blocks capable of heating microcentrifuge tubes to 56°C and 90°C*	Any vendor
Safe-Lock™ microcentrifuge tubes (1.5mL sterile, RNase/DNase free, PCR grade)	Eppendorf 022363204 or equivalent
Microcentrifuge tube racks	Any vendor
Spectrophotometer for measuring DNA concentration*	Any vendor
Vortex mixer*	Any vendor
Disposable gloves, powder-free	Any vendor
Calibrated thermometers for dry heat block*	Any vendor
Waterbath* capable of maintaining 37°C	Any vendor
Single edged blade or similar	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
cobas z 480 Analyzer
cobas ® 4800 System Control Unit with System Software version 2.1 or higher
EGFR Tissue Analysis Package Software version 1.0 or higher
Barcode Reader ext USB
Printer

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 NSCLC samples. Samples should be handled as if infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories⁹ and in the CLSI Document M29-A4.¹⁰
- **DNA PBB** and **DNA TLB** contain Triton X-100, an irritant to mucous membranes. Avoid contact with eyes, skin, and mucous membranes.
- Xylene is a hazardous chemical and should be used in a chemical hood. Discard into chemical waste in accordance with local, state, and federal regulations.
- 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® EGFR 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 and ribonuclease 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 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

- **DNA TLB, DNA EB, MGAC, EGFR MMX-1, EGFR MMX-2, EGFR MMX-3 v2, EGFR 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.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

Spillage and cleaning

- **DNA PBB** and **WB I** contain guanidine hydrochloride. If liquid containing this buffer is spilled, clean with suitable laboratory detergent and water. If a spill occurs with potentially infectious agents, clean the affected area first with laboratory detergent and water, and then with 0.5% sodium hypochlorite.
- If spills occur on the **cobas® 4800** instrument, follow the instructions in the appropriate **cobas® 4800 System - System Manual** 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 appropriate **cobas® 4800 System - System Manual**.
- For additional warnings, precautions and procedures to reduce the risk of contamination for the **cobas z 480** analyzer, consult the **cobas z 480** analyzer Instrument Manual.

Specimen collection, transport, and storage

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

Specimen collection

NSCLC FFPET specimens have been validated for use with the **cobas® EGFR Test**.

Specimen transport, storage, and stability

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

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

Processed sample storage and stability

Processed samples (extracted DNA) are stable for:

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® EGFR Mutation Test v2 workflow with cobas® DNA Sample Preparation Kit

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 microwell plate with amplification reagents
10	Load microwell plate with sample
11	Seal microwell plate
12	Load microwell 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 NSCLC FFPET sections of 5-micron thickness containing at least 10% tumor content by area are to be used in the cobas® EGFR Test. Any sample containing less than 10% tumor content by area should be macro-dissected after deparaffinization.

Note: Refer to the cobas z 480 analyzer Instrument Manual for detailed operating instructions for the cobas z 480 analyzer.

Note: Dry heat blocks capable of heating Safe-Lock™ microcentrifuge tubes should be turned on and set at 56°C and 90°C.

Run size

A single run can include from 1 to 30 samples (plus controls) per 96-well microwell plate. When running more than 24 samples, multiple cobas® EGFR Test kits will be required.

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

Reagent preparation

Prepare working reagents as shown in below prior to using the kit for the first time. Use a 5-mL serological pipette to dispense the water. Use 25-mL serological pipettes to dispense the ethanol. If the Proteinase K has already been reconstituted and frozen, thaw a sufficient number of aliquots to process the number of samples to be run.

Reagents	Reconstitution / Preparation
Proteinase K (PK)	Reconstitute Proteinase K (PK) by adding 4.5 mL of sterile, nuclease-free (PCR grade) water to the vial using a sterile, disposable 5-mL serological pipette. Mix by inverting the vial 5 to 10 times. Aliquot 450 µL of reconstituted PK into 1.5 mL Safe-Lock™ microcentrifuge tubes and store at -20°C for up to 90 days or until the expiration date, whichever comes first. If the Proteinase K has already been reconstituted and frozen, thaw sufficient number of aliquots to process the number of samples to be run prior to deparaffinization (70 µL of reconstituted PK is required for each sample).
Wash Buffer I (WB I)	Prepare working WB I by adding 15 mL of absolute ethanol to the bottle of WB I. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working WB I at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.
Wash Buffer II (WB II)	Prepare working WB II by adding 50 mL of absolute ethanol to the bottle of WB II. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working WB II at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.

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

Deparaffinization of FFPET sections mounted on slides

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

Note: If the sample contains less than 10% tumor content by area, the section must be macro-dissected.

1. Add a slide with a mounted 5-micron FFPET section to a container with sufficient xylene to cover the tissue; soak for 5 minutes.
2. Transfer the slide to a container with sufficient absolute ethanol to cover the tissue; soak for 5 minutes.
3. Remove the slide from the ethanol and allow the section to air dry completely (5 to 10 minutes).

4. Perform macro-dissection if the sample contains less than 10% tumor content by area.
5. Label one 1.5 mL Safe-Lock™ microcentrifuge tube for each sample with the sample identification information.
6. Add 180 µL **DNA TLB** to the 1.5-mL Safe-Lock™ microcentrifuge tube.
7. Add 70 µL of reconstituted PK to the Safe-Lock™ microcentrifuge tube containing **DNA TLB**.
8. Scrape the tissue off the slide and into the Safe-Lock™ microcentrifuge tube. Immerse the tissue in the **DNA TLB/PK** mixture.
9. Continue with Step 1 of the **DNA Isolation procedure**.

Deparaffinization of FFPET sections not mounted on slides

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

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

1. Place one 5-micron FFPET section into a 1.5 mL Safe-Lock™ microcentrifuge tube labeled with the sample identification information for each sample.
2. Add 500 µL Xylene to the Safe-Lock™ microcentrifuge tube containing the FFPET section.
3. Mix well by vortexing for 10 seconds.
4. Let the tube stand for 5 minutes at 15°C to 30°C.
5. Add 500 µL absolute ethanol and mix by vortexing for 10 seconds.
6. Let the tube stand for 5 minutes at 15°C to 30°C.
7. Centrifuge at 16,000 x g to 20,000 x g for 2 minutes. Remove the supernatant without disturbing the pellet. Discard the supernatant into chemical waste.
8. Add 1 mL absolute ethanol and vortex for 10 seconds.
9. Centrifuge at 16,000 x g to 20,000 x g for 2 minutes. Remove the supernatant without disturbing the pellet. Discard the supernatant into chemical waste.
10. If the pellet is floating in the remaining supernatant, spin again for 1 minute at 16,000 x g to 20,000 x g. Remove any remaining supernatant.
11. Dry the tissue pellet for 10 minutes at 56°C in a heating block with the tube open.
12. Make sure the ethanol is completely evaporated and the pellet is dry before proceeding to the next step.
13. If needed, dry pellets can be stored up to 24 hours at 2°C to 8°C.
14. Resuspend the tissue pellet in 180 µL DNA Tissue Lysis Buffer (DNA TLB).
15. Add 70 µL of reconstituted PK.
16. Continue with Step 1 of the DNA Isolation procedure.

DNA isolation procedure

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

1. Vortex the tubes containing the sample/**DNA TLB/PK** mixture and the negative control mixture (**NEG**) for 30 seconds.

Note: The tissue must be fully immersed in the DNA TLB/PK mixture.

2. Place tubes in the 56°C dry heat block and incubate for 60 minutes.
3. Vortex the tubes for 10 seconds.

Note: The tissue must be fully immersed in the DNA TLB/PK mixture.

4. Place tubes in the 90°C dry heat block and incubate for 60 minutes.

Note: During the incubation, prepare the required number of filter tubes (FTs) with hinged caps by placing the FT onto a collection tube (CT) and labeling each FT cap with the proper sample or control identification.

Note: Each sample will need 1 FT, 3 CTs and 1 elution tube (1.5 mL Safe-Lock™ microcentrifuge tube).

Note: During the incubation, label the required number of elution tubes (1.5 mL microcentrifuge tube) with the proper sample or control identification information.

5. Allow the tubes to cool to 15°C to 30°C. After cooling, pulse-centrifuge the tubes to collect liquid from the caps.
6. Add 200 µL **DNA PBB** to each tube; mix by pipetting up and down 3 times.
7. Incubate the tubes at 15°C to 30°C for 10 minutes.
8. Add 100 µL isopropanol to each tube; mix lysate by pipetting up and down 3 times.
9. Transfer each lysate into the appropriately labeled **FT/CT** unit.
10. Centrifuge the **FT/CT** units at 8,000 x g for 1 minute.
11. Place each **FT** onto a new **CT**. Discard the flow-through from the old **CT** into chemical waste, and properly dispose of the used **CT**.

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

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

13. Centrifuge the **FT/CT** units at 8,000 x g for 1 minute.
14. Discard the flow-through in each **CT** into chemical waste. Place the **FT** back into the same **CT**.
15. Add 500 µL working **WB II** to each **FT**.

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

16. Centrifuge the **FT/CT** units at 8,000 x g for 1 minute.
17. Place each **FT** onto a new **CT**. Discard the flow-through from the old **CT** into chemical waste, and properly dispose of the used **CT**.
18. Centrifuge the **FT/CT** units at 16,000 to 20,000 x g for 1 minute to dry the filter membranes.
19. Place each **FT** into an elution tube (1.5 mL microcentrifuge tube) pre-labeled with sample or control identification. Discard the flow-through from the used **CT** into chemical waste, and properly dispose of the used **CT**.
20. Add 100 µL **DNA EB** to the center of each **FT** membrane without touching the **FT** membrane.
21. Incubate the **FT** with elution tube at 15°C to 30°C for 5 minutes.
22. Centrifuge the **FT** with elution tube at 8,000 x g for 1 minute to collect eluate into the elution tube. Properly dispose of the used **FT**.
23. Close the cap on the elution tube. The elution tube contains the DNA Stock. Proceed to Step 1 in the **DNA Quantitation** section.

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

DNA quantitation

1. Mix each DNA Stock by vortexing for 5 seconds.
2. Quantify DNA using a spectrophotometer according to the manufacturer's protocol. Use **DNA EB** as the blank for the instrument. An average of two consistent readings is necessary. The two measurements should be within $\pm 10\%$ of each other when the DNA concentration readings are > 20.0 ng/ μ L. For DNA concentration readings < 20.0 ng/ μ L, the two measurements should be within ± 2 ng/ μ L. If the two measurements are not within $\pm 10\%$ of each other when the DNA concentration readings are > 20.0 ng/ μ L or within ± 2 ng/ μ L when the DNA concentration readings are < 20.0 ng/ μ L, 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.

3. The DNA Stock concentration from the samples must be > 2 ng/ μ L to perform the **cobas® EGFR Test**. Three amplification/detections are run per sample, using 25 μ L of a 2 ng/ μ L 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/ μ L to perform the **cobas® EGFR Test**. If the concentration of a DNA Stock is < 2 ng/ μ L, repeat the deparaffinization, DNA Isolation, and DNA Quantitation procedures for that sample using two 5- μ m FFPE sections. For mounted samples, after deparaffinization, combine the tissue from both sections into one tube, immerse the tissue in DNA TLB + PK, and perform DNA Isolation and Quantitation as described above. For unmounted samples, combine two sections into one tube and immerse the tissue in DNA TLB + PK, and perform DNA Isolation and Quantitation as described above. If the DNA Stock is still < 2 ng/ μ L, request another FFPE sample section from the referring clinical site.*

*Note: Processed samples (extracted DNA) are stable for up to 24 hours at 15°C to 30°C or up to 14 days at 2°C to 8°C or up to 60 days at -15°C to -25°C or 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.*

Amplification and detection

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

Instrument set-up

Refer to the **cobas z 480** analyzer Instrument Manual for detailed instruction for the **cobas z 480** set up.

Test order set-up

For detailed instructions on the EGFR workflow steps, refer to the **cobas® 4800 System cobas z 480** analyzer Instrument Manual and Software Operator's Manual for the **cobas® EGFR Mutation Test v2**.

Generate a plate map with the position of all the samples and controls in the run. The Mutant Control is loaded into positions A01 – A03 on the plate. The Negative Control is loaded into positions B01 – B03 on the plate. Diluted samples are then added in sets of 3 columns, starting from C01 – C03 through H09 – H12, as shown in Figure 2.

Figure 2 Plate layout for the cobas® EGFR Mutation Test v2

Row / Column	01	02	03	04	05	06	07	08	09	10	11	12
A	MC MMX 1	MC MMX 2	MC MMX 3 v2	S7 MMX 1	S7 MMX 2	S7 MMX 3 v2	S15 MMX 1	S15 MMX 2	S15 MMX 3 v2	S23 MMX 1	S23 MMX 2	S23 MMX 3 v2
B	NEG MMX 1	NEG MMX 2	NEG MMX 3 v2	S8 MMX 1	S8 MMX 2	S8 MMX 3 v2	S16 MMX 1	S16 MMX 2	S16 MMX 3 v2	S24 MMX 1	S24 MMX 2	S24 MMX 3 v2
C	S1 MMX 1	S1 MMX 2	S1 MMX 3 v2	S9 MMX 1	S9 MMX 2	S9 MMX 3 v2	S17 MMX 1	S17 MMX 2	S17 MMX 3 v2	S25 MMX 1	S25 MMX 2	S25 MMX 3 v2
D	S2 MMX 1	S2 MMX 2	S2 MMX 3 v2	S10 MMX 1	S10 MMX 2	S10 MMX 3 v2	S18 MMX 1	S18 MMX 2	S18 MMX 3 v2	S26 MMX 1	S26 MMX 2	S26 MMX 3 v2
E	S3 MMX 1	S3 MMX 2	S3 MMX 3 v2	S11 MMX 1	S11 MMX 2	S11 MMX 3 v2	S19 MMX 1	S19 MMX 2	S19 MMX 3 v2	S27 MMX 1	S27 MMX 2	S27 MMX 3 v2
F	S4 MMX 1	S4 MMX 2	S4 MMX 3 v2	S12 MMX 1	S12 MMX 2	S12 MMX 3 v2	S20 MMX 1	S20 MMX 2	S20 MMX 3 v2	S28 MMX 1	S28 MMX 2	S28 MMX 3 v2
G	S5 MMX 1	S5 MMX 2	S5 MMX 3 v2	S13 MMX 1	S13 MMX 2	S13 MMX 3 v2	S21 MMX 1	S21 MMX 2	S21 MMX 3 v2	S29 MMX 1	S29 MMX 2	S29 MMX 3 v2
H	S6 MMX 1	S6 MMX 2	S6 MMX 3 v2	S14 MMX 1	S14 MMX 2	S14 MMX 3 v2	S22 MMX 1	S22 MMX 2	S22 MMX 3 v2	S30 MMX 1	S30 MMX 2	S30 MMX 3 v2

Where: MC= Mutant Control, NEG = Negative Control S# = sample ID, and MMX # corresponds to Master Mix Reagent 1, 2, or 3 v2.

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 v2 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).

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

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

- For each sample, calculate the volume (μL) of DNA Specimen Diluent (DNA SD) needed:

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

Example:

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

$$1. \mu\text{L of DNA Stock} = (90 \mu\text{L} \times 2 \text{ ng}/\mu\text{L}) \div 6.5 \text{ ng}/\mu\text{L} = 27.7 \mu\text{L}$$

$$2. \mu\text{L of DNA SD} = (90 \mu\text{L} - 27.7 \mu\text{L}) = 62.3 \mu\text{L}$$

Dilution calculation for DNA stock concentrations > 36 ng/μL

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

Note: Three 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 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.

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

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

Example:

DNA stock concentration = 100 ng/ μL

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

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 Safe-Lock™ 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 Safe-Lock™ microcentrifuge tube labeled as **NEG**.
3. Vortex each DNA stock and the negative control 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 negative control (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 v2)

Note: EGFR MMX-1, EGFR MMX-2, EGFR MMX-3 v2, and working MMX are light-sensitive and must be protected from prolonged exposure to light.

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

Note: The EGFR MMX-1, EGFR MMX-2, and EGFR MMX-3 v2 may appear light blue/purplish. This does not affect the performance of the reagent.

Prepare three bulk working MMX, one containing **EGFR MMX-1**, one containing **EGFR MMX-2**, and the other containing **EGFR MMX-3 v2** in separate 1.5 mL Safe-Lock™ microcentrifuge tubes.

1. Calculate the volume of **EGFR MMX-1** or **EGFR MMX-2** or **EGFR MMX-3 v2** required for each working MMX using the following formula:

$$\text{Volume of EGFR MMX-1 or EGFR MMX-2 or EGFR MMX-3 v2 required} = (\text{Number of Samples} + 2 \text{ Controls} + 1) \times 20 \mu\text{L}$$

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

$$\text{Volume of MGAC required} = (\text{Number of Samples} + 2 \text{ Controls} + 1) \times 5 \mu\text{L}$$

Use Table 2 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 2 Volumes of reagents needed for working MMX-1, working MMX-2 and working MMX-3 v2

		# 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	5 µL	20	25	30	35	40	45	50	55	60	65
Total Vol. for Each Working MMX (µL)		100	125	150	175	200	225	250	275	300	325

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

- Remove the appropriate number of **EGFR MMX-1**, **EGFR MMX-2**, **EGFR MMX-3 v2**, 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 v2.
- Add the calculated volume of **EGFR MMX-1** or **EGFR MMX-2** or **EGFR MMX-3 v2** to their respective working MMX tube.
- Add the calculated volume of **MGAC** to the working MMX tubes.
- Vortex the tubes for 3 to 5 seconds to ensure adequate mixing.

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

Note: Use only cobas® 4800 System Microwell Plate (AD-Plate) and Sealing film.

Preparation of plate

1. Pipette 25 µL of working MMX into each reaction well of the microwell plate (AD-plate) that is needed for the run. Do not allow the pipettor tip to touch the plate outside the well.
 - Add working MMX-1 (containing **EGFR MMX-1**) to the microwell plate (AD-plate) wells in columns 01, 04, 07, and 10, as needed.
 - Add working MMX-2 (containing **EGFR MMX-2**) to the microwell plate (AD-plate) wells in columns 02, 05, 08, and 11, as needed.
 - Add working MMX-3 v2 (containing **EGFR MMX-3 v2**) to the microwell plate (AD-plate) wells in columns 03, 06, 09, and 12, as needed.
2. Pipette 25 µL of **EGFR MC** into wells **A01**, **A02**, and **A03** of the microwell plate (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 microwell plate (AD-plate); mix well using pipette to aspirate and dispense within the well a minimum of two times.

Note: Each run must contain positive control (EGFR MC) in wells A01, A02 and A03, and negative control (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 microwell plate (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 the second samples (wells D01, D02, and D03). Follow the template in Figure 2 until all samples' DNA Dilutions are loaded onto the microwell plate (AD-plate). Ensure that all liquid is collected at the bottom of the wells.
5. Cover the microwell plate (AD-plate) with sealing film (supplied with the plates). Use the sealing film applicator to seal the film firmly to the microwell plate (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® EGFR Operator's Manual for detailed instructions on the EGFR workflow steps. When the "Select test" pop-up window appears, select "EGFR Tissue 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 3.

Table 3 Result interpretation for the cobas® EGFR Test

Test Result	Mutation Result	Interpretation
Mutation Detected	Ex19Del S768I L858R T790M L861Q G719X Ex20Ins (More than one mutation may be present)	Mutation detected in specified targeted EGFR region.
No Mutation Detected (NMD)*	N/A	Mutation not detected in targeted EGFR 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.

* A “No Mutation Detected” result does not preclude the presence of a mutation in the targeted EGFR 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 “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 “Sample Dilution”, continue with “Preparation of Working Master Mix (working MMX)” and the remainder of the amplification and detection procedure.

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

Quality control and validity of results

One set of cobas® EGFR Test Mutant Control (EGFR MC) (wells A01, A02 and A03) and negative control (NEG) (wells B01, B02 and B03) for working MMX-1, working MMX-2, and working MMX-3 v2 are included in each run of up to

30 samples. A run is valid if the EGFR Mutant Control (**EGFR MC**) and the negative control (**NEG**) are valid. If an EGFR Mutant Control (**EGFR MC**) or negative control (**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 microwell plate (AD-plate) with controls for amplification and detection.

Mutant control

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

Negative control

The negative control (**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® EGFR Mutation Test v2 has only been validated for use with NSCLC FFPET tumor specimens.
2. The cobas® EGFR Mutation Test v2 has only been validated using the cobas® DNA Sample Preparation Kit (Roche P/N: 05985536190).
3. Detection of a mutation is dependent on the number of copies present in the specimen and may be affected by sample integrity, amount of isolated DNA, and the presence of interfering substances.
4. Reliable results are dependent on adequate specimen fixation, transport, storage and processing. Follow the procedures in this Package Insert and in the cobas® EGFR Test Operator's Manual.
5. The effects of other potential variables such as specimen fixation variables have not been evaluated.
6. The addition of AmpErase enzyme into the cobas® EGFR Test Master Mix enables selective amplification of target DNA; however, good laboratory practices and careful adherence to the procedures specified in this Package Insert are necessary to avoid contamination of reagents.
7. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the cobas® 4800 System.
8. Only the cobas z 480 analyzer has been validated for use with this product. No other thermal cycler with real-time optical detection can be used with this product.
9. The presence of PCR inhibitors may cause false negative or invalid results.
10. Though rare, mutations within the genomic DNA regions of the EGFR gene covered by the primers or probes used in the cobas® EGFR Test may result in failure to detect presence of a mutation in exons 18, 19, 20, and 21 (results of "No Mutation Detected").
11. The cobas® EGFR Test shows cross-reactivity (results of "Mutation Detected") to the exon 19 L747S mutation, a rare acquired mutation that may confer resistance to TKI treatment.¹²
12. The cobas® EGFR Test is validated for use with 50 ng of DNA per reaction well. DNA input amounts lower than 50 ng per reaction well are not recommended.
13. The cobas® EGFR Test is a qualitative test. The test is not for quantitative measurements of percent mutation.
14. NSCLC FFPET specimens containing degraded DNA may affect the ability of the test to detect the EGFR mutations.
15. Samples with results reported as "No Mutation Detected" may harbor EGFR mutations not detected by the assay.

16. The **cobas**[®] EGFR Test detects EGFR mutations in NSCLC patients whose tumors have the exon 18 (G719X) substitutions, exon 19 deletions, exon 20 insertions and substitutions (T790M, S768I) and exon 21 substitutions (L858R, L861Q), but not any other EGFR mutations.

Non-clinical performance evaluation

Note: The study descriptions below include cumulative data performed with v1 and v2 of the cobas® EGFR 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 NSCLC FFPET specimen was determined using a NGS method.

Analytical sensitivity – limit of blank

To assess performance of the cobas® EGFR Test in the absence of template and to ensure that a blank sample does not generate an analytical signal that might indicate a low concentration of mutation, samples with no template and NSCLC FFPET EGFR wild-type specimens were evaluated. Using the analysis prescribed in the CLSI EP17-A2E guideline¹², the Limit of Blank was determined to be zero for all mutations.

Limit of detection using FFPET specimen blends

Three FFPET specimen DNA extracts for the exon 19 deletion mutations, four FFPET specimen DNA extracts for the L858R mutation, two dual mutant FFPET specimen DNA extracts for L858R and T790M mutations, two FFPET specimen DNA extracts for the G719A mutation, one dual mutant FFPET specimen DNA extract for T790M and G719A, one dual mutant FFPET specimen DNA extract for G719C and S768I mutation, one dual mutant FFPET specimen DNA extract for S768I and G719S, three FFPET specimen DNA extracts for the exon 20 insertion mutation, and three FFPET specimen DNA extracts for the L861Q mutation were blended with EGFR wild-type FFPET specimen extracts to achieve blends with samples targeting 10, 5.0, 2.5 and 1.25% mutation level as determined by next generation sequencing method (NGS), that was validated for the use for detecting EGFR mutations in exons 18, 19, 20, and 21. Serial dilutions of each specimen blend were prepared and eight replicates of each panel member were run using each of three cobas® EGFR Test kit lots (n=24/panel member). The limit of detection of each sample was determined by the lowest amount of DNA that gave an EGFR “Mutation Detected” rate of at least 95% for the targeted mutation, shown in Table 4.

Table 4 Limit of Detection of the cobas® EGFR Test using FFPET specimen blends

EGFR Exon	EGFR Mutation Group	EGFR Nucleic Acid Sequence	Percent Mutation in the Panel Member to achieve ≥95% "Mutation Detected" Rate with 50 ng DNA input per reaction well (N=24 replicates)	COSMIC ID ¹³
18	G719X	2156 G>C	2.5	6239
		2156 G>C	4.7	6239
		2155 G>A	3.2	6252
		2155 G>T	5.6	6253
19	Exon 19 Deletion	2235_2249del15	1.4	6223
		2236_2250del15	2.5	6225
		2239_2256del18*	4.7	6255
		2240_2254del15	7.2	12369
		2240_2257del18	13.4**	12370
		2239_2248>C	2.2	12382
		2237_2255>T*	4.1	12384
		2237_2253>TTGCT*	6.3	12416
		2238_2252del15	2.4	23571
		2238_2252del15*	5.5	23571
2239_2257>GT*	6.0	Not Found		
20	T790M	2369 C>T	2.0	6240
		2369 C>T	2.4	6240
		2369 C>T	3.0	6240
	S768I	2303 G>T	1.3	6241
		2303 G>T	2.4	6241
	Exon 20 Insertion	2307_2308insGCCAGCGT G	1.7	12376
2319_2320insCAC		6.8	12377	
2310_2311insGGT		1.3	12378	
21	L858R	2573 T>G	4.0	6224
		2573 T>G	4.2	6224
		2573 T>G	4.3	6224
		2573 T>G	4.3	6224
		2573 T>G	5.3	6224
	L861Q	2582T>A	2.1	6213
		2582T>A	2.2	6213
		2582T>A	3.4	6213

* Only a single level targeting approximately 5% mutation was tested for these non-predominant exon 19 deletion mutations present in the EURTAC cohort. Specimen DNA blends were tested across 3 study sites.

** Limit of Detection of the cobas® EGFR Test for this mutation is greater than 10% mutation level using the standard input of 50 ng per reaction well.

This study demonstrates that the cobas® EGFR Test can detect mutations in EGFR exons 18, 19, 20, and 21 with at least 5% mutation level using the standard input of 50 ng per reaction well.

Minimal tumor content

A total of 66 independent EGFR mutant specimens (i.e., 35 of exon 19 deletion mutants and 31 exon 21 L858R mutants) with tumor content ranging from 25% to 99% were tested to determine the minimum tumor content required for detecting the EGFR mutation in NSCLC specimens. None of the specimens evaluated had both an exon 19 deletion mutation and an exon 21 L858R mutation. Each specimen was tested without macrodissection (neat), and after macrodissection. The observed CtR values for the neat and macrodissected slides were analyzed using

Deming regression and the Bland-Altman plot (differences vs. mean). The results support the use of specimens whose tumor content is greater than 25% without macrodissection.

An additional 10 EGFR wild-type NSCLC specimens (1-90% tumor content) and 10 EGFR mutant specimens (8-95% tumor content) were tested to determine the whether macro-dissection of low percent tumor NSCLC tumor tissue would improve detectability of the cobas® EGFR Test. Each specimen was tested without macro-dissection (neat), and after macro-dissection. All macro-dissected results matched all non-macro-dissected results and expected mutation and wild-type results were observed for all 20 specimens.

In the Phase III EURTAC trial of erlotinib vs. cisplatin-based chemotherapy, NSCLC FFPET specimens with less than 10% tumor content were macro-dissected prior to EGFR mutation analysis. A subset of the EURTAC screened specimens was evaluated for EGFR mutation status by both the cobas® EGFR Test and the next generation sequencing (NGS) methods. Table 5 and Table 6 included NSCLC specimens with valid paired results of EGFR exon 19 or L858R mutations combined from both the cobas® EGFR Test and the NGS sequencing. Using the NGS as the reference method, results showed that macro-dissection of NSCLC FFPET sections with less than 10% tumor content demonstrated comparable analytical accuracy to NSCLC FFPET section without macro-dissection.

Together, these studies support that macrodissection is required for NSCLC FFPET sections with less than 10% tumor content prior to testing with the cobas® EGFR Test.

Table 5 Performance of the cobas® EGFR Test for NSCLC FFPET specimens with tumor contents ≤10% (macro-dissected)

Measure of Agreement	Percent Agreement (N)	95% CI
Positive Percent Agreement (PPA)	97.2% (35/36)	85.8% - 99.5%
Negative Percent Agreement (NPA)	94.5% (52/55)	85.1% - 98.1%
Overall Percent Agreement (OPA)	95.6% (87/91)	89.2% - 98.3%

Table 6 Performance of the cobas® EGFR Test for NSCLC FFPET specimens with tumor contents > 10% (not macro-dissected)

Measure of Agreement	Percent Agreement (N)	95% CI
Positive Percent Agreement (PPA)	93.0% (107/115)	86.9%, 96.4%
Negative Percent Agreement (NPA)	98.5% (199/202)	95.7%, 99.5%
Overall Percent Agreement (OPA)	96.5% (306/317)	93.9%, 98.1%

Cross-Reactivity to Other Exon 18, 19, 20, and 21 Mutations

EURTAC Clinical Trial Specimens

The cobas® EGFR Test gave “Mutation Detected” results for the following EGFR mutations observed in the EURTAC clinical trial specimens (Table 7). Analytical performance of the cobas® EGFR Test in detecting these mutations has not been evaluated.

Table 7 Mutations Observed in the EURTAC Cohort Determined to Cross-React with the cobas® EGFR Test

Exon	Mutation Sequence	AA Change	COSMIC ID ¹³
19	2236_2252>AT	E746_T751>I	26680
	2239_2253>CAA	L747_T751>Q	51527
	2234_2251>AAT	K745_T751>K	Not Found
	2236_2244del9	E746_R748>E	Not Found
	2236_2263>GAAGCAT	E746_A755>E	Not Found
	2237_2251>AAC	E746_751T>E	Not Found

AURA2 Clinical Trial Specimens

The cobas® EGFR Mutation Test v2 gave “Mutation Detected” results for the following EGFR mutations observed in the AURA2 clinical trial specimens (Table 8). Analytical performance of the cobas® EGFR Mutation Test v2 in detecting these mutations has not been evaluated.

Table 8 Mutations Observed in the AURA2 Trial Determined to Cross-React with the cobas® EGFR Test

Exon	Mutation Sequence	AA Change	COSMIC ID ¹³
19	2254_2277del24	S752_I759delSPKANKEI	6256
	2236_2256>ATC	E746_S752>I	133190
21	2572_2573CT>AG	L858R	13553

Specificity – microorganisms and EGFR homologs

Specificity of the cobas® EGFR Test was evaluated by testing lung-related microorganisms, and plasmids of EGFR homologs, i.e., plasmids containing the sequences from each of the HER2, HER3, and HER4 genetic regions analogous to the sequences in EGFR exons 18, 19, 20, and 21 amplified by the cobas® EGFR Test.

Lung-related microorganisms

Streptococcus pneumoniae and *Haemophilus influenzae* at 4×10^5 colony forming units were found not to cross react or interfere with the cobas® EGFR Test when added to specimens containing wild-type and mutant EGFR sequences during the tissue lysis step.

Plasmids of EGFR homologs

Structurally related epidermal receptor tyrosine kinase protein analog sequences (EGFR/HER1, HER2, HER3 and HER4) have been shown not to cross-react with the cobas® EGFR Test when the potential cross-reactive sequence was added at a genomic copy number equivalent to 50 ng/PCR input to the isolated DNA stock prior to the amplification/detection procedure. A control condition without plasmid DNA was included. Results indicated that the observed mutations for all 20 tested FFPET specimens matched the expected mutation as determined by sequencing, in the presence and absence of the added HER gene plasmid DNA. Additionally, the EGFR exon 19 mutation L747S was tested for cross reactivity. Results indicated that the cobas® EGFR Test cross-reacts with the EGFR exon 19 mutation L747S.

Interference

Triglycerides (37 mM, CLSI recommended high concentration¹⁴) and hemoglobin (2 mg/mL, CLSI recommended high concentration¹⁴) have been shown not to interfere with the cobas® EGFR Test when the potential interfering substance was added to the lysis step during the specimen preparation procedure.

Albuterol (Ventolin), Ipratropium (Atrovent), Fluticasone (Flonase), Ceftazidime (Fortaz), Imipenem-cilastin (Primaxin), Piperacillin-tazobactam, Cilastin (Cilastatin sodium), Betadine and Lidocaine were shown to not interfere with the performance of the cobas® EGFR Test when added to the lysis step during the specimen preparation procedure.

Necrotic tissue

NSCLC FFPET specimens with necrotic tissue content up to 60% for EGFR mutant and 85% in wild-type specimens have been shown not to interfere with the call results using the cobas® EGFR Test.

Repeatability

Repeatability of the cobas® EGFR Test was assessed using six FFPET specimens, including: two EGFR wild-type specimens; four EGFR mutant specimens, one of each: exon 19 deletion, S768I and G719X, T790M and L858R, and exon 20 insertion mutations. These specimens were tested in duplicate by two operators, using two different reagent lots and two cobas z 480 analyzers over four days. A total of 32 replicates were evaluated per sample. The cobas® EGFR Test had a correct call rate of 96.9% (186/192).

Repeatability of the cobas® EGFR Test was also assessed in a second study using four FFPET specimens including: one EGFR wild-type specimens; three EGFR mutant FFPET specimens, one of each: L861Q, G719X, and exon 20 insertion mutations. The specimens were tested in duplicate by two operators, using two different reagent lots and two cobas z 480 analyzers over multiple days. The cobas® EGFR Test has a correct call accuracy of 99.2% (127/128) across all specimen replicates, operators, reagent lots, and instruments combined.

Specimen handling reproducibility

The reproducibility of the cobas® DNA Sample Preparation Kit was examined using sections taken from three FFPET specimen blocks, one containing an exon 19 deletion mutation, one containing an L858R mutation, and one that is wild-type. Each specimen was tested in duplicate at each site on each day. The specimen sections for a given specimen were randomized and tested over a six day period across three sites using one operator at each site, one cobas z 480 analyzer at each site, three cobas® DNA Sample Preparation Kit lots, and one cobas® EGFR Test kit lot. On each test day, each operator isolated and tested the DNA from two NSCLC FFPET curl sections for each specimen using the cobas® EGFR Test. All specimens reported valid and correct results through-out the six days of testing. For all specimens and operators combined, the cobas® EGFR Test had a correct call rate of 100% (108/108).

Clinical performance evaluation

Clinical reproducibility study 1

An external study was performed to assess the reproducibility of the cobas® EGFR Test across 3 external testing sites (2 operators per site), 3 reagent lots, and 5 non-consecutive testing days, with a 13-member panel of DNA samples extracted from FFPET sections of NSCLC Wild Type (WT) and Mutant type (MT) tumor specimens. This panel included the L858R mutation in exon 21 and five different exon 19 deletion mutations. Of 92 runs, 90

(97.8%) were valid. A total of 2,340 tests were performed on the 13 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 10 of the 12 MT panel members. For panel member EX19_2240_2257del18 - 5% Mutation, agreement was 62.8% (67 of 180 test results were Mutation Not Detected). For panel member EX19_2240_2257del18 - 10% Mutation, agreement was 99.4% (1 of 180 test result was Mutation Not Detected). Results by overall agreement are presented in Table 9. The coefficient of variation (CV) was < 6% in all mutation panel members. Within each panel member, the CV was < 3.5%. For external control the overall CV was < 1.3%. The CV% was < 0.5% for between lots and < 1.2% for within-lot.

Table 9 Overall agreement estimates by panel member in the cobas® EGFR Test v2 reproducibility study 1

Panel Member	Number of Valid Tests	Agreement (N)	Agreement % (95% CI) ^a
Wild Type	180	180	100 (98.0, 100.0)
EX19_2235_2249del15 - 5% Mutation	180	180	100 (98.0, 100.0)
EX19_2235_2249del15 - ≤10% Mutation	180	180	100 (98.0, 100.0)
EX19_2236_2250del15 - 5% Mutation	180	180	100 (98.0, 100.0)
EX19_2236_2250del15 - ≤10% Mutation	180	180	100 (98.0, 100.0)
EX19_2239_2248>C - 5% Mutation	180	180	100 (98.0, 100.0)
EX19_2239_2248>C - ≤10% Mutation	180	180	100 (98.0, 100.0)
EX19_2240_2254del15 - 5% Mutation	180	180	100 (98.0, 100.0)
EX19_2240_2254del15 - ≤10% Mutation	180	180	100 (98.0, 100.0)
EX19_2240_2257del18 - 5% Mutation	180	113	62.8 (55.3, 69.9)*
EX19_2240_2257del18 - ≤10% Mutation	180	179	99.4 (96.9, 100.0)*
EX21_2573T>G=L858R - 5% Mutation	180	180	100 (98.0, 100.0)
EX21_2573T>G=L858R - ≤10% Mutation	180	180	100 (98.0, 100.0)

Note: Results were in agreement when a Mutant Type panel member had a valid result of “Mutation Detected” or when Wild Type panel member had a valid result of Mutation Not Detected.

^a 95% CI = 95% exact binomial confidence interval.

* Analytical sensitivity of the cobas® EGFR Test for detecting this mutation is greater than 10% mutation level using the standard input of 50 ng per reaction well.

Clinical Reproducibility Study 2

An external study was performed to assess the reproducibility of the cobas® across 3 external testing sites (2 operators per site), 3 reagent lots, and 5 non-consecutive testing days, with an 11-member panel of DNA samples extracted from FFPE sections of NSCLC Wild Type (WT) and mutant tumor specimens. This panel included the exon 18 G719X mutation, exon 20 T790M mutation, exon 20 S768I mutation, exon 20 insertion mutation, and exon 21 L861Q mutation. Of 91 runs, 90 (98.9%) were valid. A total of 1,980 tests were performed with 11 panel members tested in duplicate 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 all mutant panel members, except for the Exon 20 Insertion LOD panel member. Results by overall agreement are presented in Table 10 below. The coefficient of variation (CV) was <9.2% in all mutant panel members. For the external control, the overall CV was ≤1.3%. The CV was ≤0.6% between lots and ≤1.1% within-lot.

Table 10 Overall Agreement Estimates by Panel Member in the cobas® EGFR Test Reproducibility Study 2

Panel Member	Number of Valid Tests	Agreement (N)	Agreement % (95% CI)*
Wild Type	180	180	100 (98.0, 100.0)
Exon 18 G719X - LOD	180	180	100 (98.0, 100.0)
Exon 20 T790M - LOD	180	180	100 (98.0, 100.0)
Exon 20 S768I - LOD	180	180	100 (98.0, 100.0)
Exon 20 Insertion - LOD	180	166	92.2 (87.3, 95.7)
Exon 21 L861Q - LOD	180	180	100 (98.0, 100.0)
Exon 18 G719X - 2X LOD	180	180	100 (98.0, 100.0)
Exon 20 T790M - 2X LOD	180	180	100 (98.0, 100.0)
Exon 20 S768I - 2X LOD	180	180	100 (98.0, 100.0)
Exon 20 Insertion - 2X LOD	180	180	100 (98.0, 100.0)
Exon 21 L861Q - 2X LOD	180	180	100 (98.0, 100.0)

Note: Results were in agreement when a Mutant Type panel member had a valid result of mutation detected for the target mutation or when a Wild Type panel member had a valid result of NMD.

* 95% CI = 95% exact binomial confidence interval.

CI = confidence interval; LOD = limit of detection; NMD = No Mutation Detected

Correlation to reference method using Phase III samples from EURTAC trial

The clinical performance of the cobas® EGFR Test was assessed by comparing it to two reference methods – 2x bidirectional Sanger sequencing and quantitative next generation sequencing (NGS) – using 487 formalin-fixed paraffin-embedded lung tumor specimens from patients with advanced NSCLC who were screened in the Phase III EURTAC trial of erlotinib vs. cisplatin-based chemotherapy.^{6,7} The clinical and demographic characteristics of the patients whose specimens were available for this retrospective testing were comparable to those of otherwise eligible patients (557) whose specimens were not available for retesting.

A total of 1,276 patients were screened for the EURTAC trial using a combination of laboratory developed tests, collectively referred to as the clinical trial assay (CTA). After excluding ineligible patients and those without CTA results, 1,044 patients were potentially eligible for the current study. Among the 1,044 eligible patients, 225 patients had samples that were mutation positive by CTA, 792 had samples that were Wild Type by CTA, and 27 had samples with inconclusive results by CTA. Of the 1,044 potentially eligible patients, 487 specimens were available for retesting with the cobas® EGFR Test.

All 487 specimens were tested in a blinded fashion with both the cobas® EGFR Test and Sanger sequencing. Of those, 406 had valid results by both the cobas® EGFR Test and Sanger sequencing, 38 invalid results were observed by the cobas® EGFR Test and Sanger sequencing, 38 invalid results by Sanger sequencing only, and 5 invalid results by the cobas® EGFR Test only. Among the 487 specimens available for retesting with the cobas® EGFR Test, 444 specimens gave valid cobas® EGFR Test results and were also tested with NGS. Of those, there were 36 invalid results by NGS; thus, 408 had valid results by both the cobas® EGFR Test and NGS. The analytical accuracy of the cobas® EGFR Test compared with each reference method was evaluated by estimating the positive percentage agreement (PPA), negative percentage agreement (NPA), and overall percentage agreement (OPA) and their corresponding 95% CIs for exon 19 deletions and L858R mutations in aggregate, and separately.

In the EURTAC cohort, the cobas® EGFR Test detected mutations in exon 19 and exon 21 of the EGFR gene as listed in Table 11. Of the mutations detected in the EURTAC cohort, analytical sensitivity was demonstrated on the mutations listed in Table 4.

Table 11 Mutations Detected by the cobas® EGFR Test in the EURTAC Cohort

Exon	Mutation Sequence	AA Change	COSMIC ID ¹³
19	2235_2249del15	E746_A750delELREA	6223
	2236_2250del15	E746_A750delELREA	6225
	2239_2256del18	L747_S752delLREATS	6255
	2240_2254del15	L747_T751delLREAT	12369
	2240_2257del18	L747_P753>S	12370
	2239_2248>C	L747_A750>P	12382
	2239_2251>C	L747_T751>P	12383
	2237_2255>T	E746_S752>V	12384
	2237_2253>TTGCT	E746_T751>VA	12416
	2237_2257>TCT	E746_P753>VS	18427
	2238_2252del15	L747_T751delLREAT	23571
	2236_2252>AT	E746_T751>I	26680
	2239_2253>CAA	L747_T751>Q	51527
	2234_2251>AAT	K745_T751>K	Not Found
	2236_2244del9	E746_R748>E	Not Found
	2236_2263>GAAGCAT	E746_A755>E	Not Found
	2237_2251>AAC	E746_751T>E	Not Found
2239_2257>GT	L747_P753>V	Not Found	
21	2573 T>G	L858R	6224

A total of 406 samples with valid cobas® EGFR Test and Sanger results were included in the agreement analysis. The PPA between the cobas® EGFR Test and Sanger sequencing was 96.6% (95% CI: 91.5% to 98.7%), and the NPA was 88.3% (95% CI: 84.1% to 91.5%), in the detection of exon 19 deletions and L858R mutations in aggregate as presented in Table 12. The OPA was 90.6%, with the lower limit of the 95% CI above 87%. The PPA, NPA, and OPA in the detection of exon 19 deletion mutations were all >92%. The PPA, NPA, and OPA in the detection of L858R mutations compared were all >95%.

Table 12 Comparison of the cobas® EGFR Test with Sanger sequencing for the detection of EGFR exon 19 deletion mutations and L858R mutation

Mutation	Measure of Agreement	Percent Agreement (N)	95% CI
Exon 19 Deletion	Positive Percent Agreement (PPA)	97.3% (71/73)	90.5%, 99.2%
	Negative Percent Agreement (NPA)	92.5% (308/333)	89.2%, 94.9%
	Overall Percent Agreement (OPA)	93.3% (379/406)	90.5%, 95.4%
L858R	Positive Percent Agreement (PPA)	95.3% (41/43)	84.5%, 98.7%
	Negative Percent Agreement (NPA)	97.5% (354/363)	95.4%, 98.7%
	Overall Percent Agreement (OPA)	97.3% (395/406)	95.2%, 98.5%
Aggregate	Positive Percent Agreement (PPA)	96.6% (112/116)	91.5%, 98.7%
	Negative Percent Agreement (NPA)	88.3% (256/290)	84.1%, 91.5%
	Overall Percent Agreement (OPA)	90.6% (368/406)	87.4%, 93.1%

A total of 408 samples with valid cobas® EGFR test and NGS results were included in the agreement analysis. By comparison, the PPA and NPA between the cobas® EGFR Test and NGS for the detection of exon 19 deletions and

the L858R point mutation in aggregate were 94.0% (95% CI: 89.1% to 96.8%) and 97.7% (95% CI: 95.0% to 98.9%), respectively as presented in Table 13. The OPA was 96.3%, with a lower limit of the 95% CI of 94.0%. The PPA, NPA, and OPA in detecting exon 19 deletion mutations were all >95%, with all the 95% lower limit CIs >90%. The PPA, NPA, and OPA in detecting the L858R mutation were also all >95%, with all lower limits of the 95% CIs \geq 95% except for PPA (90%), owing to due to the small number of L858R mutation detected.

Table 13 Comparison of the cobas® EGFR Test with NGS for the detection of EGFR exon 19 deletion mutations and L858R mutation in aggregate

Mutation	Measure of Agreement	Percent Agreement (N)	95% CI
Exon 19 Deletion	Positive Percent Agreement (PPA)	95.9% (94/98)	90.0%, 98.4%
	Negative Percent Agreement (NPA)	99.7% (309/310)	98.2%, 99.9%
	Overall Percent Agreement (OPA)	98.8% (403/408)	97.2%, 99.5%
L858R	Positive Percent Agreement (PPA)	90.6% (48/53)	79.7%, 95.9%
	Negative Percent Agreement (NPA)	98.6% (350/355)	96.7%, 99.4%
	Overall Percent Agreement (OPA)	97.5% (398/408)	95.5%, 98.7%
Aggregate	Positive Percent Agreement (PPA)	94.0% (142/151)	89.1%, 96.8%
	Negative Percent Agreement (NPA)	97.7% (251/257)	95.0%, 98.9%
	Overall Percent Agreement (OPA)	96.3% (393/408)	94.0%, 97.8%

Correlation to Reference Method using Phase II Samples from AURA2

The clinical performance of the cobas® EGFR Test was assessed by comparing it with a validated next generation sequencing (NGS) platform using 383 formalin-fixed paraffin-embedded lung tumor specimens from patients with advanced NSCLC who were screened using the cobas® EGFR Mutation Test in the Phase II AURA2 trial of Tagrisso®.

A total of 472 patients were screened for the AURA2 trial using the cobas® EGFR Test. After excluding ineligible patients, 383 patients were potentially eligible for the current study.

All 383 specimens were tested in a blinded fashion with both the cobas® EGFR Test and a validated NGS method. Of those, 368 had valid results by both the cobas® EGFR Test and NGS, A total of 2 invalid results were observed by both the cobas® EGFR Test and NGS, 2 invalid results by NGS only, and 11 invalid results by the cobas® EGFR Test only. The analytical accuracy of the cobas® EGFR Test compared with the reference method, NGS, for detection of the T790M mutation was evaluated by estimating the positive percent agreement (PPA), negative percent agreement (NPA), and overall percent agreement (OPA), and their corresponding 95% CIs for the T790M mutation.

In the AURA2 trial, the cobas® EGFR Test detected the mutations of the EGFR gene as listed in Table 14. Of the mutations detected in the AURA 2 trial, analytical sensitivity was demonstrated on the mutations listed in Table 4.

Table 14 Mutations Detected by the cobas® EGFR Test in the AURA 2 Cohort

Exon	Mutation Sequence	AA Change	COSMIC ID ¹³
18	2156G>C	G719A	6239
	2155G>A	G719S	6252
	2155G>T	G719C	6253
19	2239_2247delTTAAGAGAA	L747_E749delLRE	6218
	2235_2249del15	E746_A750delELREA	6223
	2236_2250del15	E746_A750delELREA	6225
	2239_2256del18	L747_S752delLREATS	6255
	2240_2254del15	L747_T751delLREAT	12369
	2240_2257del18	L747_P753>S	12370
	2239_2248>C	L747_A750>P	12382
	2239_2251>C	L747_T751>P	12383
	2237_2255>T	E746_S752>V	12384
	2237_2251del15	E746_T751>A	12678
	2235_2248>AATTC	E746_A750>IP	13550
	2235_2252>AAT	E746_T751>I	13551
	2253_2276del24	S752_I759delSPKANKEI	13556
2237_2257>TCT	E746_P753>VS	18427	
20	2369C>T	T790M	6240
	2303G>T	S768I	6241
21	2573T>G	L858R	6224
	2573_2574TG>GT	L858R	12429
	2582T>A	L861Q	6213

A total of 368 samples with valid cobas® EGFR Test and NGS results were included in the agreement analysis. The PPA between the cobas® EGFR Test and NGS was 88.3% (95% CI: 83.8% to 91.7%), the NPA was 97.3% (95% CI: 92.4% to 99.1%), and the OPA was 91.0% (95% CI: 87.7% to 93.5%) for the detection of the T790M mutation as presented in Table 15. Thirty samples were positive by NGS but negative by the cobas® EGFR Test: in 11/30 samples, the percent T790M mutation determined by NGS was below LoD (< 2% mutation) of the cobas® EGFR Test. In 19/30 samples, a moderately delayed IC Ct value indicate poor amplifiability of the DNA template.

Table 15 Comparison of the cobas® EGFR Test with NGS for the Detection of the EGFR T790M Mutation

Measure of Agreement	Percent Agreement (N)	95% CI
Positive Percent Agreement (PPA)	88.3% (226/256)	83.8%, 91.7%
Negative Percent Agreement (NPA)	97.3% (109/112)	92.4%, 99.1%
Overall Percent Agreement (OPA)	91.0% (335/368)	87.7%, 93.5%

Clinical outcome data

EURTAC

The EURTAC trial was a Phase III, multicenter, open-label, randomized study of Tarceva® (erlotinib) versus standard platinum doublet chemotherapy as first-line therapy in chemotherapy-naïve patients with advanced NSCLC whose tumors harbor EGFR exon 19 deletions or exon 21 (L858R) substitution mutations, as assessed by a clinical trial assay (CTA). The study was conducted under the sponsorship of the Spanish Lung Cancer Group (SLCG). A total number of 174 patients were enrolled into the study. The trial results showed that patients who received Tarceva® had a statistically significant increase in progression-free survival (PFS) (median PFS 10.4

months vs. 5.1 months) as compared to patients who received chemotherapy, with a hazard ratio of 0.34 ($p < 0.0001$, 95% CI [0.21; 0.54]). The response rate of patients on the Tarceva® arm was greater than the response rate of patients treated with chemotherapy (65.1% vs. 16.1%). No significant difference was observed in overall survival (OS) in the two arms, as 76% of patients on the standard chemotherapy arm crossed over to receive Tarceva®.

Of the 174 patients enrolled into the EURTAC trial, 134 cases (77% of the study population, including 69 patients from the erlotinib arm and 65 patients from the chemotherapy arm) were available for retesting and tested retrospectively by the cobas® EGFR Test. Of the 134 cobas® EGFR Test retested cases, 116 cases (59 patients from the erlotinib arm and 57 patients from the chemotherapy arm) were “Mutation Detected” by the cobas® EGFR Test. Analysis of the 116 subset revealed that those patients treated with Tarceva® had a significant increase in PFS time (median PFS 10.4 vs. 5.4 months and less likely to have an event of progressive disease or death (HR= 0.34, 95% CI [0.21;0.54], $p < 0.0001$) than patients treated with chemotherapy (Figure 3). The response rate in the Tarceva® arm was greater compared to the chemotherapy arm (59.3% vs. 14.0%). No significant difference in OS was observed between the two groups. The observed clinical benefit in the subset of patients tested with the cobas® EGFR Test was comparable to that observed in the full study population (Table 16).

Additional efficacy analysis was conducted to consider patients who were tested positive by the cobas® EGFR Test but were tested negative or invalid by the CTA. In the worst case scenario (assuming a hazard ratio of 1 for patients positive by the cobas® EGFR Test and negative by CTA), data demonstrated a hazard ratio of 0.42 (95% CI [0.26; 0.57]).

Figure 3 Kaplan-Meie (investigator

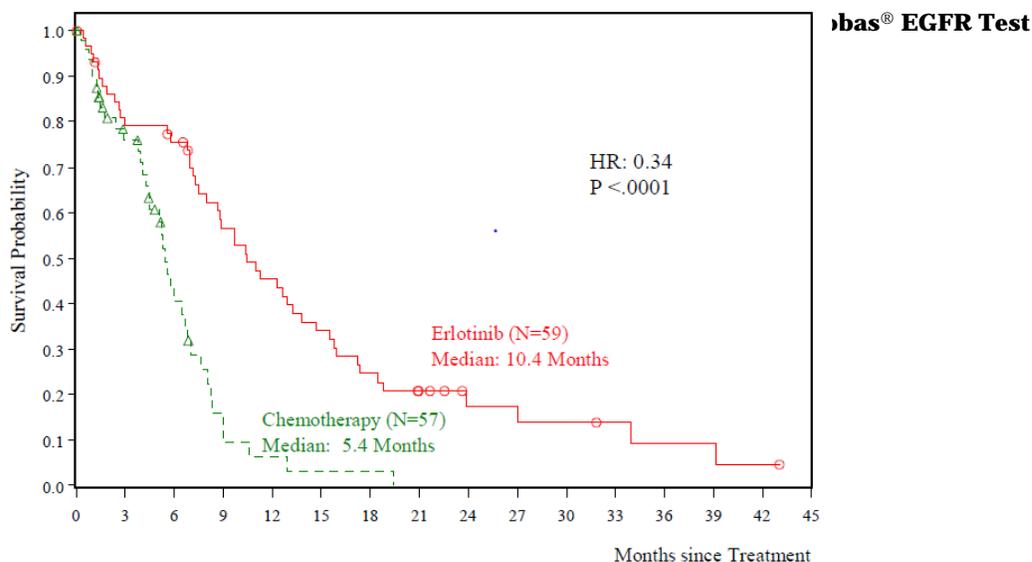


Table 16 Clinical benefit of patients tested with the cobas® EGFR Test is comparable to that observed in the EURTAC population

Parameter	cobas®-positive Population n = 116		EURTAC n = 173*	
	Chemotherapy n = 57	Erlotinib n = 59	Chemotherapy n = 87	Erlotinib n = 86
PFS				
Median (Months)	5.4	10.4	5.1	10.4
Hazard Ratio	0.34		0.34	

Hazard Ratio 95% CI	[0.21; 0.54]	[0.23; 0.49]
P-Value (log-rank test)	<0.0001	<0.0001

*Note: One patient withdrew consent after completion of the EURTAC study, which resulted in a dataset of n = 173

AURA2

The AURA2 trial was a Phase II, multicenter, open-label, single-arm study, assessing the safety and efficacy of Tagrisso® as a second or ≥third-line therapy in patients with advanced NSCLC, who had progressed following prior therapy with an approved EGFR TKI agent. All patients were required to have T790M mutation-positive NSCLC as detected by the cobas® EGFR Test. The primary efficacy outcome measure was objective response rate (ORR) according to RECIST 1.1 as evaluated by Blinded Independent Central Review (BICR) using the evaluable for response analysis set. The ORR was defined as the number (%) of patients with at least 1 visit response of complete response (CR) or partial response (PR) that was confirmed at least 4 weeks later (ie, a best objective response [BOR] of CR or PR).

Of the 472 patients screened for the AURA2 trial, 383 patients were eligible for testing with the cobas® EGFR Test. Of those eligible, 233 T790M+ patients were recruited into the AURA2 trial, and 210 patients were enrolled and received Tagrisso® (full analysis set [FAS]).

All 383 patients eligible for AURA2 trial, were retested by the cobas® EGFR Mutation Test v2. Of 233 T790M positive patients recruited into the AURA 2 trial, 225 were T790M+ by the cobas® EGFR Mutation Test v2 and 204 were in the FAS. Of 204 patients who received at least one dose of Tagrisso®, 193 ERAS had measurable disease confirmed by BICR (ERAS). Of 193 patients, 126 were confirmed responders by BICR with an ORR of 65.3% (95% CI: 58.1% to 72.0%).

Of 204 patients who received Tagrisso®(FAS), 127 were confirmed responders by BICR with an ORR of 62.3% (95% CI: 55.2% to 68.9%) and 133 by investigator assessment with an ORR of 65.2% (95% CI: 58.2% to 71.7%).

Table 17 below presents the ORR by BICR and investigator assessment in AURA2. Of 198 patients who received at least one dose of Tagrisso® and had measurable disease confirmed by BICR (ERAS), 127 were confirmed responders by BICR with an ORR of 64.1% (95% CI: 57.0% to 70.8%). Of 210 patients who received at least one dose of Tagrisso® (FAS), 128 were confirmed responders by BICR with an ORR of 61.0% (95% CI: 54.0% to 67.6%) and 135 by investigator assessment with an ORR of 64.3% (95% CI: 57.4% to 70.8%).

All 383 patients eligible for AURA2 trial, were retested by the cobas® EGFR Mutation Test v2. Of 233 T790M positive patients recruited into the AURA 2 trial, 225 were T790M+ by the cobas® EGFR Mutation Test v2 and 204 were in the FAS. Of 204 patients who received at least one dose of Tagrisso®, 193 ERAS had measurable disease confirmed by BICR (ERAS). Of 193 patients, 126 were confirmed responders by BICR with an ORR of 65.3% (95% CI: 58.1% to 72.0%).

Of 204 patients who received Tagrisso® (FAS), 127 were confirmed responders by BICR with an ORR of 62.3% (95% CI: 55.2% to 68.9%) and 133 by investigator assessment with an ORR of 65.2% (95% CI: 58.2% to 71.7%).

Table 17 Clinical Benefit of T790M+ Patients Tested With The cobas® EGFR Test in the AURA2 Trial

Analysis Set	Assessed by	AURA2			cobas EGFR Mutation Test v2 T790M Positive		
		N	Number of Confirmed Responders	ORR (95% CI)	N	Number of Confirmed Responders	ORR (95% CI)
Full Analysis Set (FAS)	Blinded Independent Central Review	210	128	61.0% (54.0%, 67.6%)	204	127	62.3% (55.2%, 68.9%)

		AURA2			cobas EGFR Mutation Test v2 T790M Positive		
Analysis Set	Assessed by	N	Number of Confirmed Respon- ders	ORR (95% CI)	N	Number of Confirmed Respon- ders	ORR (95% CI)
	Investigator		135	64.3% (57.4%, 70.8%)		133	65.2% (58.2%, 71.1%)

Additional information

Symbols

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

Table 18 Symbols used in labeling for Roche PCR diagnostic products



Ancillary Software



In Vitro Diagnostic Medical Device



Authorized Representative
in the European community



Lower Limit of Assigned Range



Barcode Data Sheet



Manufacturer



Batch code



Store in the dark



Biological Risks



Contains Sufficient for $\langle n \rangle$ tests



Catalogue number



Temperature Limit



Consult instructions for use



Test Definition File



Contents of kit



Upper Limit of Assigned Range



Distributed by



Use-by date



For IVD Performance Evaluation
Only



Global Trade Item Number



This product fulfills the requirements of the European Directive 98/79 EC for *in vitro* diagnostic medical devices.

Manufacturer and distributors

Table 19 Manufacturer and distributors



Roche Molecular Systems, Inc.
1080 US Highway 202 South
Branchburg, NJ 08876 USA



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

Trademarks and patents

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

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