cobas® EGFR Mutation Test v2

For in vitro diagnostic use

IVD

cobas® DNA Sample Preparation Kit	24 Tests	P/N: 05985536190
cobas® cfDNA Sample Preparation Kit	24 Tests	P/N: 07247737190
cobas® EGFR Mutation Test v2	24 Tests	P/N: 07248563190

TABLE OF CONTENTS

cobas® EGFR Mutation Test v2 : Intended Use

The **cobas**® EGFR Mutation Test v2 is a real-time PCR test for the qualitative detection and identification of mutations in exons 18, 19, 20, and 21 of the epidermal growth factor receptor (EGFR) gene in DNA derived from formalin-fixed paraffin-embedded (FFPET) tumor tissue or plasma from non-small cell lung cancer (NSCLC) patients. The test also is intended to be used as an aid in selecting patients with NSCLC for therapy with an EGFR tyrosine kinase inhibitor (TKI).

The **cobas**® EGFR Mutation Test v2 for use with plasma is further indicated for the semi-quantitative measurement of mutations in exons 18, 19, 20, and 21 of the EGFR gene from serial collections of human plasma as an aid in the management of NSCLC cancer patients.

FFPET samples are processed using the **cobas**® DNA Sample Preparation Kit and plasma samples are processed using the **cobas**® cfDNA Sample Preparation Kit. The **cobas**® EGFR Mutation Test v2 and **cobas z** 480 analyzer are used together 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 in exon 20, insertion mutations in exon 20, and L858R and L861Q substitution mutations in exon 21.

Table 1 lists the EGFR mutations detected by the **cobas**® EGFR Test.

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 sample preparation to obtain DNA from FFPET or plasma; 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

The **cobas**® DNA Sample Preparation Kit and the **cobas**® cfDNA Sample Preparation Kit are manual sample preparations based on nucleic acid binding to glass fibers. A protease and chaotropic lysis/binding buffer releases nucleic acids and protects the released 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 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 target DNA is then amplified and detected on the **cobas z** 480 analyzer using the amplification and detection reagents provided in the **cobas**® EGFR Mutation Test v2 kit.

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. 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. Four 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 four 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).[7] 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. The **cobas**® EGFR Test has been demonstrated to inactivate deoxyuridine-containing EGFR mutant amplicon.

FOLLOW INSTRUCTIONS IN SECTION A FOR USE WITH TISSUE SAMPLES.
FOLLOW INSTRUCTIONS IN SECTION B FOR USE WITH PLASMA SAMPLES.

SECTION A: FOR USE WITH TISSUE SAMPLES

Sample Preparation

FFPET samples are processed and genomic DNA isolated using the **cobas**® DNA Sample Preparation Kit, a manual sample preparation based on nucleic acid binding to glass fibers. A deparaffinized 5-micron 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**® EGFR Mutation Test v2 kit.

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	 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.
	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

[a] Product safety labeling primarily follows EU GHS guidance

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 -20ºC. 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 15ºC to 30ºC. 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 2ºC to 8ºC 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 56ºC and 90ºC*	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 37ºC	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 (e.g. HP P2055d)

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 Laboratories[8] and in the CLSI Document M29-A4.[9]
- **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.[10]

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 the table 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 ±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 negative control (NEG CT) does not need to be measured.

3. The DNA Stock concentration from the samples must be > 2 ng/µL to perform the **cobas**® EGFR 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® EGFR Mutation Test. If the concentration of a DNA Stock is < 2 ng/µL, repeat the deparaffinization, DNA Isolation, and DNA Quantitation procedures for that sample using two 5 µm FFPET sections. For mounted samples, after deparaffinization, combine the tissue from both sections into one tube, immerse the tissue in DNA TLB + PK, and perform DNA Isolation and Quantitation as described above. For unmounted samples, combine two sections into one tube and immerse the tissue in DNA TLB + PK, and perform DNA Isolation and Quantitation as described above. If the DNA Stock is still < 2 ng/µL, request another FFPET 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.

Instrument set-up

1. Turn on the **cobas z** 480 analyzer. The instrument will take several minutes to warm up before the run can begin.
2. Turn on the control unit. The control unit logs into Windows automatically.
3. Double click on the **cobas**® 4800 software icon and log on to perform the run using the specified lab user ID and password.
4. Click on the "New Run" icon from the menu.
5. "Select Test" pop up window appears. Select EGFR Tissue Test and click the "OK" button.
6. When the "Work Place" Screen appears, type or scan in the MWP Barcode in the Microwell Plate ID field. Scan the barcodes for the DNA Isolation kit ID and the **cobas**® EGFR Mutation Test Reagent kit ID in the appropriate fields. In the "Sample" field, enter "24" for the first and "6" for the second kit (if you are intending to run a full plate of 30 samples). If you intend to run fewer than 24 samples, then enter the appropriate number in the sample field in the first row.
7. The "Sample ID" is automatically filled in for your control positions. Type or scan in your unique "Sample ID" for every sample into the Sample ID column.
8. Upon completion of entering the "Sample IDs", select "Save" button located at the bottom left hand corner of the screen.
9. Save the file with the default file name assigned by the software.

10. Generate a plate layout with the position of all the samples and controls in the run by clicking the Print button and selecting File -> Print in the Preview window. Always fill the plate column-wise, starting with the EGFR MC positions A01 – A03 and the EGFR NEG in positions B01 – B03. Map the samples beginning with positions C01 – C03 and continuing down to H01 – H03, then proceeding to positions A04 - A06 down to H04 - H06 until all samples have been mapped (Figure 2).

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 Specimen Diluent (**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 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:

   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 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:

   Volume of **EGFR MMX-1** or **EGFR MMX-2** or **EGFR MMX-3 v2** 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 5 µL

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

3. 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.
4. Add the calculated volume of **EGFR MMX-1** or **EGFR MMX-2** or **EGFR MMX-3 v2** 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 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 DNA from each sample and 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.

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 (MMX-1, MMX-2 and MMX-3 v2)" 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 FFPET 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.[11]
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 metastatic 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 guideline[12], the Limit of Blank was determined to be zero for all mutation classes.

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[6]
18	G719X	2155 G>T	5.6	6253
		2155 G>A	3.2	6252
		2156 G>C	4.7	6239
		2156 G>C	2.5	6239
19	Exon 19 Deletion	2235_2249del15	1.4	6223
		2236_2250del15	2.5	6225
		2238_2252del15	2.4#	23571
		2239_2248>C	2.2	12382
		2240_2254del15	7.2	12369
		2240_2257del18	13.4**	12370
		2237_2253>TTGCT*	6.32	12416
		2237_2255>T*	4.08	12384
		2239_2256del18*	4.74	6255
		2238_2252del15*	5.45#	23571
		2239_2257>GT*	6.02	Not Found
20	T790M	2369 C>T	2.4	6240
		2369 C>T	3.0	6240
		2369 C>T	2.0	6240
	S768I	2303 G>T	2.4	6241
		2303 G>T	1.3	6241
	Exon 20 Insertion	2307_2308insGCCAGCGTG	6.8	12376
		2310_2311insGGT	1.3	12378
		2319_2320insCAC	2.1	12377
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.

#Two independent specimens for the exon 19 deletion (2238_2252del15) were tested.

+Five independent L858R specimens were tested.

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%

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 x 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 concentration[13]) and hemoglobin (2 mg/mL, CLSI recommended high concentration[13]) 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

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 7. 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 7 Overall agreement estimates by panel member in the cobas® EGFR Test reproducibility study

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.

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 3 EURTAC trial of erlotinib vs. cisplatin-based chemotherapy.[14,15] 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.

In the EURTAC cohort, the **cobas®** EGFR Test detected the following deletion mutations in exon 19 of the EGFR gene (Table 8).

Table 8 Mutations detected in the EURTAC cohort with the cobas® EGFR Test

Exon	Mutation	AA Change	COSMIC ID[6]
19	2234_2251>AAT	K745_T751>K	Not Found
	2236_2244del9	E746_R748>E	Not Found
	2236_2252>AT	E746_T751>I	26680
	2236_2263>GAAGCAT	E746_A755>E	Not Found
	2237_2251>AAC	E746_751T>E	Not Found
	2239_2253>CAA	L747_T751>Q	51527
	2237_2257>TCT	E746_P753>VS	18427
	2239_2251>C	L747_T751>P	12383

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 9. The OPA was 90.6%, with the lower limit of the 95% CI above 87%.

Table 9 Comparison of the cobas® EGFR Test with Sanger sequencing for the detection of EGFR exon 19 deletion mutations and L858R mutation

Measure of Agreement	Percent Agreement (N)	95% CI
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 10. The OPA was 96.3%, with a lower limit of the 95% CI of 94.0%.

Table 10 Comparison of the cobas® EGFR Test with NGS for the detection of EGFR exon 19 deletion mutations and L858R mutation in aggregate

Measure of Agreement	Percent Agreement (N)	95% CI
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%

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 [0.23; 0.49]). 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 11).

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-Meier plot of PFS by treatment for patients with mutation detected by the cobas® EGFR Test (investigator assessment)

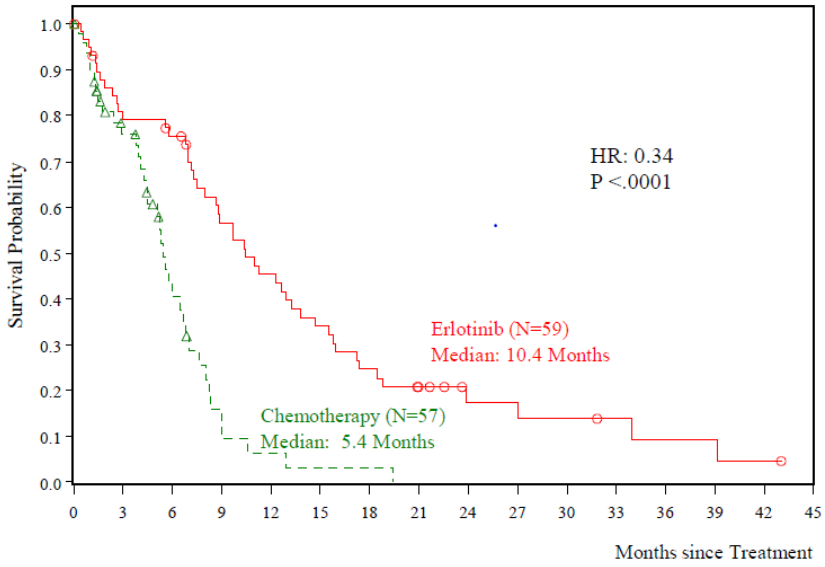


Table 11 Clinical benefit of patients tested with the cobas® EGFR Test is comparable to that observed in the EURTAC population

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

SECTION B: FOR USE WITH PLASMA SAMPLES

Sample Preparation

Plasma samples are processed and circulating cell free DNA (cfDNA) isolated using the **cobas**® cfDNA Sample Preparation Kit, a generic manual sample preparation based on nucleic acid binding to glass fibers. Two milliliters (mL) of plasma are processed with a protease and chaotropic binding buffer that protects the cfDNA from DNases. Subsequently, isopropanol is added to the binding mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the cfDNA is bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and then eluted with an aqueous solution. 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 Mutation Test v2 kit.

Materials and reagents

Materials and reagents provided

Kit/Cassettes	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning[a]
cobas® **cfDNA Sample Preparation Kit** 24 Tests (P/N: 07247737190)	**PK** (Proteinase K) (P/N: 05860695102) Proteinase K, lyophilized	2 x 100 mg	 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.
	DNA PBB (DNA Paraffin[b] Binding Buffer) (P/N: 05517621001) Tris-HCl buffer 49.6% Guanidine hydrochloride 0.05% Urea 17.3% Triton X-100	8 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	
	HPEA FT (High Pure Extension Assembly Unit) (P/N: 07323204102) Filter tubes with caps	5 x 5 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 Kit 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: 07248601001) 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 Kit 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

[a] Product safety labeling primarily follows EU GHS guidance.

[b] Paraffin Binding Buffer is used for plasma samples.

Reagent storage and handling

Reagent	Storage Temperature	Storage Time
cobas® cfDNA Sample Preparation Kit	15°C to 30°C	Once opened and reconstituted, the PK reagent is stable for up to 30 days or until the expiration date indicated, whichever comes first. Once opened and reconstituted, the remaining cfDNA Sample Preparation kit reagents are stable for up to 90 days or until the expiration date indicated, whichever comes first.
cobas® EGFR Mutation Test v2*	2°C to 8°C	Once reagent is opened, the kit is 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.

***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 2ºC to 8ºC 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
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 pipette tips	Any vendor
Pipet-Aid™*	Drummond 4-000-100 or equivalent
Table top centrifuge* (capable of 6,000 x g while holding 50-mL conical tubes in a swing-bucket rotor)	Eppendorf model 5810or equivalent
Bench top microcentrifuge* (capable of 20,000 x g)	Eppendorf 5430 or 5430R or equivalent
15-mL Sterile conical plastic tubes	Any vendor
Microcentrifuge tubes (1.5-mL RNase/DNase free/ PCR grade)	Life Technologies AM12400 or Eppendorf 022364120 or equivalent
Conical and microcentrifuge tube racks	Any vendor
Vortex mixer*	Any vendor
Disposable powder-free gloves	Any vendor

* All equipment should be properly maintained according to 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 Plasma Analysis Package Software version 1.0 or higher
Barcode Reader ext USB
Printer (e.g. HP P2055d)

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.
- All samples should be handled as if infectious using good laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories[8] and in the CLSI Document M29-A4.[9]
- **DNA PBB** contains Triton X-100, an irritant to mucous membranes. Avoid contact with eyes, skin, and mucous membranes.
- The use of sterile disposable pipettes and DNase-free pipette 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 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.

Sample collection, transport, and storage

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

Sample collection and handling

The **cobas**® cfDNA Sample Preparation Kit has been developed for use with K2 EDTA Plasma samples.

Plasma should be separated from blood within 4 hours of collection and stored at ≤ -70°C until tested.

Sample transport, storage and stability

Plasma samples can be transported frozen. Transportation of Plasma samples must comply with country, federal, state, and local regulations for the transport of etiologic agents.[10]

Plasma Sample Storage Temperature	**≤ -70°C**	**2°C to 8°C**
Storage Time	Up to 12 months	Up to 3 days

Processed sample storage and stability

Processed sample (extracted DNA) is 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 1 freeze thaw over 60 days	Up to 7 days	Up to 1 day

Extracted DNA should be used within the recommended storage periods or before the expiration date of the **cobas**® cfDNA Sample Preparation Kit used to extract the DNA, whichever comes first.

Prior to using extracted, stored DNA stocks, pulse vortex and centrifuge the elution tube containing the stock.

Test procedure

Running the test

Figure 4 cobas® EGFR Mutation Test v2 workflow with cobas® cfDNA Sample Preparation kit

1	Start the system
2	Perform instrument maintenance
3	Remove samples and reagents from storage
4	Prepare samples for binding to column
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 K2 EDTA Plasma samples are to be used with the cobas® EGFR Test.

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

Run size

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

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

Reagent preparation and storage

Prepare working reagents as shown in the table 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 **PK** by adding 4.5 mL of sterile water to the vial using a sterile, disposable 5-mL serological pipette. Mix by inverting the vial 5 to 10 times. Aliquot 1.1 mL of reconstituted **PK** into 1.5-mL microcentrifuge tubes and store at -20°C for up to 30 days or until the expiration date, whichever comes first. If the **PK** has already been reconstituted and frozen, thaw sufficient number of aliquots to process the number of samples to be run (250 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 to 37°C until the precipitate dissolves. Do not use until all precipitate has been dissolved.

DNA isolation procedure

1. Label a 15-mL conical tube for each plasma sample and a negative control. Sterile water can serve as a negative control and can be processed the same way as samples.
2. Vortex plasma, then transfer 2 mL of each plasma sample or negative control (sterile water) to a separate 15-mL tube.

Note: A minimum of 2 mL of plasma is required to process a sample with the cobas® cfDNA Sample Preparation Kit.

3. Add 250 µL **PK** to each tube.
4. Add 2 mL of **DNA PBB** to each tube.
5. Mix the sample tubes containing **DNA PBB/PK** by inverting 3 to 5 times.
6. Incubate each tube at room temperature (15°C to 30°C) for 30 minutes.

Note: During the incubation, prepare the required number of HPEA FT by labeling each HPEA FT with proper identification on the cap of each HPEA FT.

Note: Each sample will need one HPEA FT, three collection tubes (CT) and two elution tubes (1.5-mL microcentrifuge tubes).

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

7. Add 500 µL isopropanol and mix lysate by inverting 3 to 5 times.
8. Transfer all of the lysate into the appropriately labeled **HPEA FT**.
9. Using table top centrifuge with a swing-bucket rotor, centrifuge **HPEA FT** at 4,000 x g for 5 minutes.

10. After centrifugation, remove the **HPEA FT** from the 50-mL conical collection tube. Place the **HPEA FT** onto a **CT**. Remove the larger locking clip by twisting and pulling it away from the assembly.
11. Remove the smaller locking clip from underneath the filter tube (**FT**) cap by pushing it up so that the seal is broken on both sides of the cap and then pulling it away from the assembly.
12. Remove the **HPEA** from the **FT** by tilting the extender away from the cap side of the **FT.**
13. Discard the flow-through from the **HPEA FT** into chemical waste and properly dispose of the unit.
14. Label the filter cap appropriately.
15. Add 500 µL working **WB I** to each **FT**.

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

16. Use benchtop microcentrifuge for the rest of the protocol.
17. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.
18. Place each **FT** onto a new **CT**. Discard the flow-through in each **CT** into chemical waste and properly dispose of old **CT**.
19. Add 500 µL working **WB II** to each **FT**.

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

20. Centrifuge **FT/CT** units at 8,000 x g for 1 minute.
21. Place each **FT** onto a new **CT**. Discard the flow-through from the old **CT** into chemical waste and properly dispose of the old **CT**.
22. Centrifuge **FT/CT** units at 16,000 x g – 20,000 x g for 1 minute to dry the filter membrane.
23. Place the **FT** onto an elution tube (1.5-mL RNase/DNase-free microcentrifuge tube) pre-labeled with sample identification information and put an orientation mark on each tube. Discard any flow-through in each **CT** into chemical waste and properly dispose of the old **CT**.
24. Add 100 µL **DNA EB** to the center of the **FT** membrane without touching the **FT** membrane.
25. Incubate **FT** with elution tube at room temperature (15°C to 30°C) for 5 minutes.
26. Place the tubes in the centrifuge with the orientation marks facing outward. Centrifuge FT with elution tube at 8,000 x g for 1 minute to collect eluate into the elution tube (pre-labeled 1.5-mL RNase/DNase-free microcentrifuge tube). The eluate is the DNA stock.
27. Discard the FT.
28. Slowly remove 80 µL of DNA stock, being careful not to disrupt the pellet (which may not be visible). Transfer removed DNA stock to a second elution tube (1.5-mL RNase/DNase-free microcentrifuge tube) pre-labeled with sample identification information. Close the caps on the elution tubes. DNA stock is ready for PCR tests. Store DNA stock according to instructions in **Sample transport storage and stability** section.

*Note: **If the pellet is disrupted, return the DNA stock to the original elution tube , cap the tube, then pulse vortex the tube and, with the orientation mark facing outward, centrifuge the tube at 8,000 x g for 1 minute to collect eluate and repeat step 28 to remove 80 µL of DNA stock.***

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 5.

Figure 5 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.

Instrument set-up

1. Turn on the **cobas z** 480 analyzer. The instrument will take several minutes to warm up before the run can begin.
2. Turn on the control unit. The control unit logs into Windows automatically.
3. Double click on the **cobas**® 4800 software icon and log on to perform the run using the specified lab user ID and password.
4. Click on the "New Run" icon from the menu.
5. "Select Test" pop up window appears. Select EGFR Plasma Test and click the "OK" button.
6. When the "Work Place" Screen appears, type or scan in the MWP Barcode in the Microwell Plate ID field. Scan the barcodes for the DNA Isolation kit ID and the **cobas**® EGFR Mutation Test Reagent kit ID in the appropriate fields. In the "Sample" field, enter "24" for the first and "6" for the second kit (if you are intending to run a full plate of 30 samples). If you intend to run fewer than 24 samples, then enter the appropriate number in the sample field in the first row.
7. The "Sample ID" is automatically filled in for your control positions. Type or scan in your unique "Sample ID" for every sample into the Sample ID column.
8. Upon completion of entering the "Sample IDs", select "Save" button located at the bottom left hand corner of the screen.
9. Save the file with the default file name assigned by the software.
10. Generate a plate layout with the position of all the samples and controls in the run by clicking the Print button and selecting File -> Print in the Preview window. Always fill the plate column-wise, starting with the **EGFR MC** in positions A01 – A03 and the **NEG** control in positions B01 – B03. Map the samples beginning with positions C01 – C03 and continuing down to H01 – H03, then proceeding to positions A04 - A06 down to H04 - H06 until all samples have been mapped (Figure 5).

Reaction set-up

Preparation of working master mix (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 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:

   Volume of **EGFR MMX-1** or **EGFR MMX-2** or **EGFR MMX-3 v2** 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 5 µL

Use Table 12 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 12 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

3. 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.
4. Add the calculated volume of **EGFR MMX-1** or **EGFR MMX-2** or **EGFR MMX-3 v2** 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 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

Note: If using stored DNA stocks, follow the instructions in Sample transport storage and stability section.

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 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 DNA from each sample and follow the template in Figure 5 until all samples' DNA are loaded onto the microwell plate (AD-plate). Ensure that all liquid is collected at the bottom of the wells.

Note: Prior to using stored DNA stocks, pulse vortex and centrifuge the elution tube containing the stock.

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.

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 13.

Table 13 Result interpretation for the cobas® EGFR Test

Test Result	Mutation Result	Semi-Quantitative Index (SQI) Result	Interpretation
Mutation Detected	Ex19Del S768I L858R T790M L861Q G719X Ex20Ins (More than one mutation may be present)	Ex19Del: *SQI* S768I: *SQI* L858R: *SQI* T790M: *SQI* L861Q: *SQI* G719X: *SQI* Ex20Ins: *SQI* (More than one mutation may be present)	Mutation detected in specified targeted EGFR region.
No Mutation Detected (NMD)*	N/A	N/A	Mutation not detected in targeted EGFR regions
Invalid	N/A	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	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 concentration of mutant sequences, adequate sample integrity, absence of inhibitors, and sufficient DNA to be detected.

Semi Quantitative Index (SQI)

The SQI is a semi-quantitative measure of the amount of mutant cfDNA in a sample that can be used to measure differences in mutation load over time. An increase in the SQI value indicates an increase in the amount of the

corresponding target mutation within an individual sample source, whereas a decrease in the SQI value indicates a decrease in the overall amount of the corresponding target mutation within an individual sample source.

Retesting of samples with invalid results

1. If the run is invalid, there will be insufficient volume of extracted DNA for each sample to repeat Amplification and Detection. Repeat the entire test procedure for all samples, starting with DNA isolation.
2. If the run is valid but the sample is invalid, there will be insufficient volume of extracted DNA for each sample to repeat Amplification and Detection. Repeat the entire test procedure for the invalid sample, starting with DNA isolation.

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.

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. The **cobas**® EGFR Mutation Test v2 was verified with K2 EDTA Plasma samples.
2. The **cobas**® EGFR Mutation Test v2 performance was verified using the **cobas**® cfDNA Sample Preparation Kit (Roche P/N: 07247737190) with K2 EDTA Plasma samples.
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 transport, storage and processing. Follow the procedures in these Instructions for Use and in the **cobas**® EGFR Operator's Manual.
5. Pipetting from the bottom of the elution tube may disrupt the pellet and adversely affect test results.
6. The addition of AmpErase enzyme into the **cobas**® EGFR Mutation Test v2 Master Mix enables selective amplification of target DNA; however, good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary to avoid contamination of reagents.
7. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas**® 4800 System.
8. Only the **cobas z** 480 analyzer has been validated 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. Though rare, mutations within the genomic DNA regions of the EGFR gene cover ed by the primers or probes used in the **cobas**® EGFR Test may result in failure to detect the presence of a mutation in exons 18, 19, 20, and 21 (results of "Mutation Not Detected").
11. The presence of PCR inhibitors may cause false negative or invalid results.
12. Samples tested outside the linear range of the assay may generate false results.

13. The **cobas**® EGFR Mutation Test v2 was verified for use with 25 µL of DNA stock per reaction well. DNA stock input volumes lower than 25 µL per reaction well are not recommended.
14. The procedure described above must be followed to detect ≥ 100 copies of mutant DNA per mL of K2 EDTA plasma for the EGFR mutations in Table 1.
15. Samples with results reported as "No Mutation Detected" may harbor EGFR mutations not detected by the assay.
16. Consideration should be made for a "No Mutation Detected" result in plasma to reflex to or be confirmed by tissue testing.
17. 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.[11]

Non-clinical performance evaluation

The following data is intended to demonstrate the analytical performance of the **cobas**® EGFR Test.

Limit of detection using cell line DNA

Cell line DNAs containing each of the seven mutation classes detected by the test were added to healthy-donor K2 EDTA plasma that is wild-type for EGFR. Serial dilutions were prepared and 24 replicates of each panel member were tested, using each of three **cobas**® EGFR Test kit lots.

Limit of Detection was determined for each the seven mutation classes detected by the test as the lowest concentration of DNA that gave an EGFR "Mutation Detected" rate of at least 95% for the targeted mutation. The results are shown in Table 14.

Table 14 Limit of detection of cobas® EGFR Test with K2 EDTA Plasma

EGFR Exon	EGFR Mutation	Target Nucleic Acid Sequence	Intact* DNA LOD (copies/mL)	Sheared** DNA LOD (copies/mL)
18	G719A	2156G>C	100	100
19	Ex19Del	2235_2249del15	25	75
20	S768I	2303G>T	20	25
20	T790M	2369C>T	25	100
20	Ex20Ins	2307_2308ins9GCCAGCGTG	80	25
21	L858R	2573T>G	10	100
21	L861Q	2582T>A	30	30

The differences in observed LOD are due to the difference in background DNA.

*Intact cell line DNA had a WT DNA background of approximately 10,000 copies/mL.

**Cell line DNA, mechanically sheared to an average size of 200bp, had a WT DNA background of approximately 100,000 copies/mL.

This study demonstrates that the **cobas**® EGFR Test can detect mutations in EGFR exons 18, 19, 20, and 21 with ≤ 100 copies of mutant DNA per mL of plasma using the standard input of 25 µL of DNA stock per reaction well.

Correlation to MiSeq using clinical K2 EDTA plasma samples

In order to evaluate the ability of the test to correctly identify EGFR Mutations in plasma, comparison testing of 74 K2 EDTA samples from Non-Small Cell Lung Cancer (NSCLC) patients using the **cobas**® EGFR Test and the Illumina MiSeq Sequencing Platform (MiSeq) was performed (Table 15).

Table 15 cobas® EGFR Mutation Test v2 vs. MiSeq sequencing

Measure of Agreement	Percent Agreement (n)	95% CI
Positive Percent Agreement (PPA)	80.0% (28/35)	70.3 – 83.7%
Negative Percent Agreement (NPA)	94.9% (37/39)	83.1 – 98.6%
Overall Percent Agreement (OPA)	87.8% (65/74)	81.2 – 90.7%

Linearity

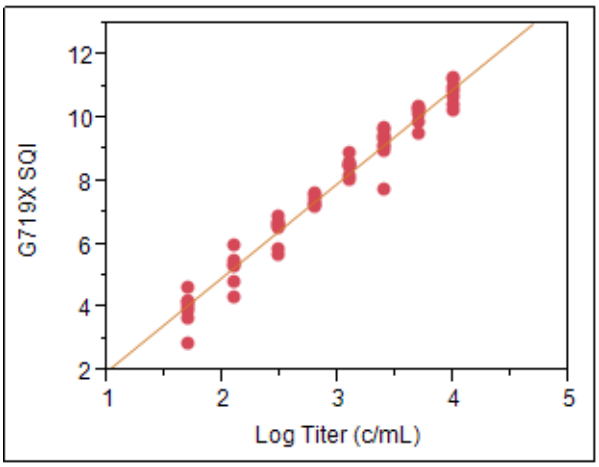
The linearity study of **cobas®** EGFR Test was performed with dilution series of at least 8 panel members spanning the linear range for the predominant mutation for each EGFR mutation class reported by the test. Panel members were prepared by diluting cell line DNAs containing each of predominant mutations into healthy-donor K2 EDTA plasma that is wild-type for EGFR. The evaluation was performed according to CLSI Guideline EP06-AE.[16] Ten replicates per panel member for each of 2 lots were tested for concentrations up to 1.0E+04 copies/mL (20 total replicates per level). Above 1.0E+04 copies/mL, one replicate per lot was tested.

For each mutation class of the **cobas®** EGFR Test, the linear range is indicated in Table 16 and the corresponding graphs for one lot are shown in Figure 6 through Figure 12.

Table 16 Linear range of the cobas® EGFR Test with K2 EDTA Plasma

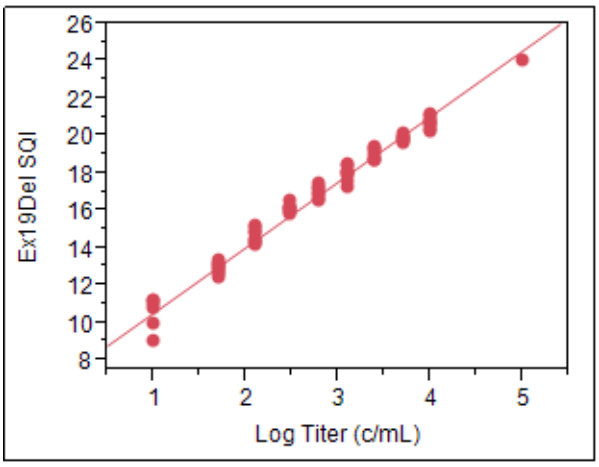
EGFR Exon	EGFR Mutation	Target Nucleic Acid Sequence	Linear Range (copies/mL)
18	G719A	2156 G>C	50 - 1E+04
19	Exon 19 Deletion	2235_2249del15	10 - 1E+05
20	S768I	2303G>T	10 - 1E+05
20	T790M	2369C>T	50 - 1E+05
20	Exon 20 Insertion	2307_2308ins9GCCAGCGTG	10 - 1E+05
21	L858R	2573T>G	10 - 1E+05
21	L861Q	2582T>A	10 - 1E+05

Figure 6 Linearity of mutant DNA in K2 EDTA Plasma: G719A cell line DNA



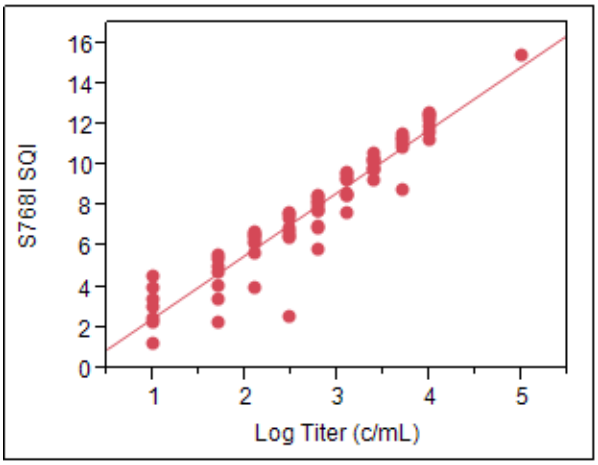
SQI = -0.987 + 2.986 * Log Copies per mL
R^2 = 0.968

Figure 7 Linearity of mutant DNA in K2 EDTA Plasma: Ex19 Del cell line DNA



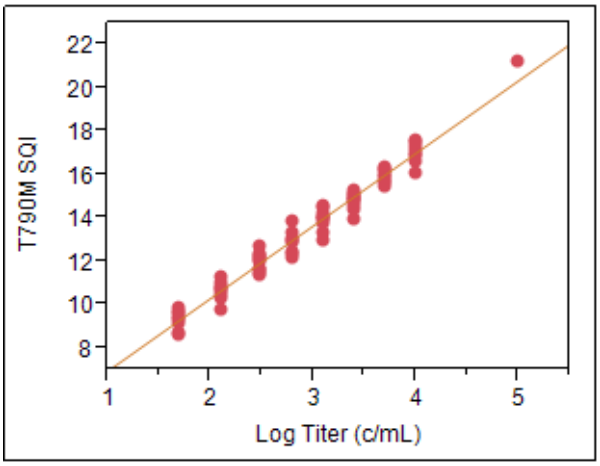
SQI = 7.042 + 3.507 * Log Copies per mL
R^2 = 0.981

Figure 8 Linearity of mutant DNA in K2 EDTA Plasma: S768I cell line DNA



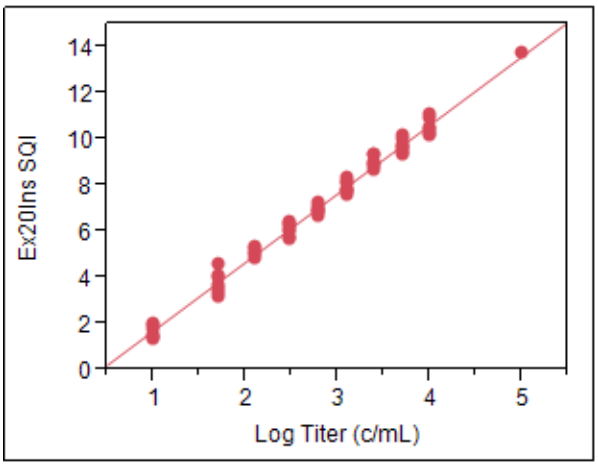
SQI = -0.578 + 3.093 *Log Copies per mL
R^2 = 0.912

Figure 9 Linearity of mutant DNA in K2 EDTA Plasma: T790M cell line DNA



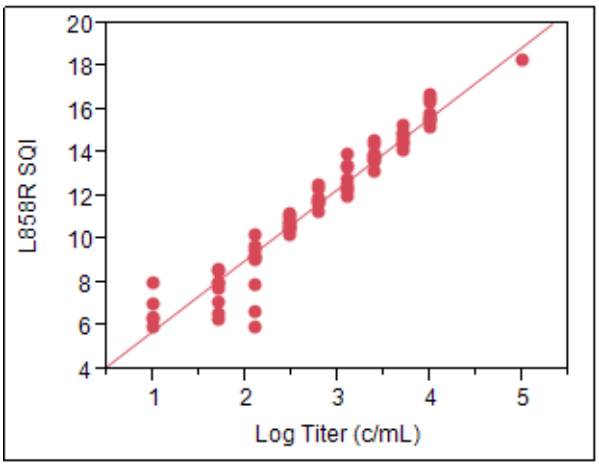
SQI = 3.593 + 3.352 *Log Copies per mL
R^2 = 0.973

Figure 10 Linearity of mutant DNA in K2 EDTA Plasma: Ex20Ins cell line DNA



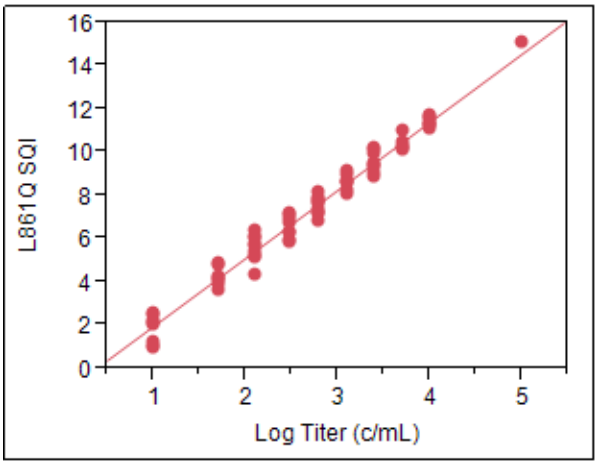
SQI = -1.268 + 2.973 *Log Copies per mL
R^2 = 0.990

Figure 11 Linearity of mutant DNA in K2 EDTA Plasma: L858R cell line DNA



SQI = 2.543 + 3.283 * Log Copies per mL
R^2 = 0.933

Figure 12 Linearity of mutant DNA in K2 EDTA Plasma: L861Q cell line DNA



SQI = -1.177 + 3.149 * Log Copies per mL
R^2 = 0.980

Repeatability

Repeatability of the **cobas**® EGFR Test was assessed using dilutions of EGFR Mutant cell line DNA diluted in healthy donor K2 EDTA plasma samples. The predominant mutation for each class reported by the test were co-diluted and assessed at 3x each mutation's respective LoD (in copies/mL), 1.0E+03 copies/mL, and 5.0E+04 copies/mL. In addition one wild-type sample was tested. Each of the four samples was tested in duplicate by two operators, using two different reagent lots and two **cobas z** 480 analyzers over 4 days. The **cobas**® EGFR Test had a correct call rate of 99.2% (381/384).

Table 17 lists the mean SQI and the SQI SD from the repeatability study.

Table 17 Mean SQI and the SQI SD from the repeatability study

EGFR Exon	EGFR Mutation	Target Nucleic Acid Sequence	Concentration (copies/mL)	Mean SQI	SD SQI (n=32)
18	G719A	2156G>C	3.00E+02	4.53	0.41
			1.00E+03	6.86	0.38
			5.00E+04	11.81	0.67
19	Ex19Del	2235_2249del15	7.50E+01	13.42	0.46
			1.00E+03	16.85	0.42
			5.00E+04	22.31	0.55
20	S768I	2303G>T	6.00E+01	5.99	0.45
			1.00E+03	8.49	0.43
			5.00E+04	14.13	0.43
20	T790M	2369C>T	7.50E+01	9.00	1.03
			1.00E+03	13.28	0.43
			5.00E+04	19.52	0.57
20	Ex20Ins	2307_2308ins9GCCAGCGTG	2.40E+02	4.92	0.43
			1.00E+03	6.77	0.40
			5.00E+04	12.61	0.60
21	L858R	2573T>G	1.20E+02	9.81	0.47
			1.00E+03	12.91	0.28
			5.00E+04	17.21	0.81
21	L861Q	2582T>A	4.50E+01	3.58	0.73
			1.00E+03	7.91	0.45
			5.00E+04	10.06	0.60

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 <*n*> 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.		

US Customer Technical Support 1-800-526-1247

Manufacturer and distributors

Table 19 Manufacturer and distributors

Manufactured in the United States
Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim, Germany

Roche Diagnostics (Schweiz) AG
Industriestrasse 7
6343 Rotkreuz, Switzerland

Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim, Germany

Roche Diagnostics, SL
Avda. Generalitat, 171-173
E-08174 Sant Cugat del Vallès
Barcelona, Spain

Roche Diagnostica Brasil Ltda.
Av. Engenheiro Billings, 1729
Jaguaré, Building 10
05321-010 São Paulo, SP Brazil

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
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Document revision

Document Revision Information	
Doc Rev. 1.0 08/2015	First Publishing.
Doc Rev. 2.0 03/2016	Added clarification on handling DNA stock/eluate, centrifuge rotor and loading the AD-plate. Please contact your local Roche Representative if you have any questions.