cobas® EGFR Mutation Test v2

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

cobas® cfDNA Sample Preparation Kit	24 Tests	P/N: 07247737190
cobas® EGFR Mutation Test v2	24 Tests	P/N: 07248555190

TABLE OF CONTENTS

Non-clinical performance evaluation

Additional information

Summary and explanation of the test

Principles of the procedure

The **cobas**®EGFR Mutation Test v2 (**cobas**®EGFR Test) is a research use only real-time PCR test for the qualitative detection and semi-quantitative measurement of defined mutations of the epidermal growth factor receptor (EGFR) gene. Defined EGFR mutations are detected using DNA isolated from circulating cell-free DNA (cfDNA) from plasma derived from EDTA anti-coagulated peripheral whole blood.

The **cobas**®EGFR Test is based on two major processes: (1) manual sample preparation to obtain DNA from 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 (defined as the combination of a deletion and an insertion)
- 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

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 Test 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 exon 19 deletion mutations, sequences ranging from 125 to 141 base pairs are targeted; for the L858R substitution mutation in exon 21, a 138 base pair sequence is targeted; for the T790M substitution mutation in exon 20, a 118 base pair sequence is targeted; for the G719X substitution mutation in exon 18, sequences ranging from 104-106 base pairs are targeted; for the S768I substitution mutation in exon 20, a 133 base pair sequence is targeted; for the exon 20 insertion mutations, sequences ranging from 125 to 143 base pairs are 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 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, the 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).[1] The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine but not DNA containing thymidine. Deoxyuridine is not present in naturally occurring DNA but is always present in amplicon due to the use of dUTP in 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.

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. P304 + P340 +P312: IF INHALED: Remove person to fresh air and keep comfortable for breathing. Call a POISON CENTER/doctor if you feel unwell. 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/doctor. P342 + P311: If experiencing respiratory symptoms: Call a POISON CENTER/doctor.
	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: 07248555190)	**EGFR MMX-1** (EGFR Master Mix 1) (P/N: 06496466001) 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: 06496474001) 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 Kit** 24 Tests (P/N: 07248555190)	**MGAC** (Magnesium acetate) (P/N: 06496539001) Magnesium acetate 0.09% Sodium azide	6 x 0.2 mL	N/A
	EGFR MC (EGFR Mutant Control) (P/N: 06496504001) 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: 06496512001) 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, stable for 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.

*** 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)	Any vendor
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 4,000 x g while holding 50 mL conical tubes in a swing bucket rotor)	Eppendorf model 5810 or equivalent.
Bench top microcentrifuge* (capable of 20,000 x g)	Eppendorf 5430 or 5430R or equivalent.
15-mL Sterile conical plastic tubes	Any vendor
Locking-lid microcentrifuge tubes (1.5-mL RNase/DNase free/ PCR grade)	Any vendor
Conical and microcentrifuge tube racks	Any vendor
Vortex mixer*	Any vendor
Disposable powder-free gloves	Any vendor

* All equipment should be 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 RUO Analysis Package Software version 1.0 or higher
Barcode Reader ext USB
Printer

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

Precautions and handling requirements

Warnings and precautions

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

- For Research Use Only. Not for use in diagnostic procedures.
- Safety Data Sheets (SDS) are available upon request from your local Roche office.
- This test is for use with plasma samples. Samples should be handled as if infectious using good laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories[2] and in the CLSI Document M29-A4.[3]
- **DNA PBB** contains Triton X-100, an irritant to mucous membranes. Avoid contact with eyes, skin, and mucous membranes.
- **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.

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.*

- **DNA EB** contains 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.
- 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 as indicated below 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.[4]

Plasma specimens are stable for either:

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 one of the following:

Extracted DNA Storage Temperature	-15°C to -25°C	2°C to 8°C	15°C to 30°C
Storage Time	Up to 2 freeze thaws over 60 days	Up to 21 days	Up to 7 days

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 1 cobas® EGFR Test 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 90 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 a 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 For Research Use Only.

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 H10 – H12, as shown in Figure 2.

Figure 2 Plate layout for the cobas® EGFR Test

Row /Column	01	02	03	04	05	06	07	08	09	10	11	12
A	MC MMX 1	MC MMX 2	MC MMX 3 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.*

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

Table 1 Volumes of reagents needed for working MMX-1, working MMX-2 and working MMX-3 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 **EGFR MC** in wells A01, A02 and A03, and **NEG** in wells B01, B02, and B03 or the run will be invalidated by the **cobas z** 480 analyzer.*

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

4. Using new pipettor tips for each 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 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. When the "Select test" pop-up window appears, select "EGFR Plasma RUO" and click the "OK" button.

Results

Interpretation of results

Note: *All run and sample validation is performed by the* ***cobas***® *4800 software.*

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

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

Table 2 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 MC** and the **NEG** are valid. If an **EGFR MC** or **NEG** is invalid, the entire run is invalid and must be repeated.

Mutant control

The **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 **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 Test has been verified for use with K2 EDTA plasma samples.
2. The **cobas**® EGFR Test performance was verified using the **cobas**® **cf**DNA Sample Preparation Kit (Roche P/N: 07247737190).
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 Test Master Mix enables selective amplification of target DNA; however, good laboratory practices and careful adherence to the procedures specified in these Instructions for Use are necessary to avoid contamination of reagents.
7. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas**® 4800 System.
8. Only the **cobas z** 480 analyzer has been verified for use with this product. No other thermal cycler with real-time optical detection can be used with this product.
9. Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to another, users perform method correlation studies in their laboratory to qualify technology differences.
10. 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 the presence of a mutation in exons 18, 19, 20, and 21 (results of "No Mutation Detected").
11. The presence of PCR inhibitors may cause false negative or invalid results.
12. The **cobas**®EGFR Test shows cross-reactivity (results of "Mutation Detected") to the exon 19 L747S mutation, a rare acquired mutation.[5]
13. Samples tested at high concentrations ($>10^5$ copies/mL) may generate false results.
14. The **cobas**® EGFR Test 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.
15. 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 3.
16. Samples with results reported as "No Mutation Detected" may harbor EGFR mutations not detected by the assay.

Table 3 Mutations detected by the cobas® EGFR Test

EGFR Exon	EGFR Mutation Reported	Mutation Sequence	AA Change	COSMIC ID[6]
Exon 18	G719X	2156 G>C	G719A	6239
		2155 G>A	G719S	6252
		2155 G>T	G719C	6253
Exon 19	Ex19Del	2240_2251del12	L747_T751>S	6210
		2239_2247del9	L747_E749del	6218
		2238_2255del18	E746_S752>D	6220
		2235_2249del15	E746_A750del	6223
		2236_2250del15	E746_A750del	6225
		2239_2253del15	L747_T751del	6254
		2239_2256del18	L747_S752del	6255
		2237_2254del18	E746_S752>A	12367
		2240_2254del15	L747_T751del	12369
		2240_2257del18	L747_P753>S	12370
		2239_2248TTAAGAGAAG>C	E747_A750>P	12382
		2239_2251>C	L747_T751>P	12383
		2237_2255>T	E746_S752>V	12384
		2235_2255>AAT	E746_S752>I	12385
		2237_2252>T	E746_T751>V	12386
		2239_2258>CA	L747_P753>Q	12387
		2239_2256>CAA	L747_S752>Q	12403
		2237_2253>TTGCT	E746_T751>VA	12416
		2238_2252>GCA	L747_T751>Q	12419
		2238_2248>GC	L747_A750>P	12422
		2237_2251del15	E746_T751>A	12678
		2236_2253del18	E746_T751del	12728
		2235_2248>AATTC	E746_A750>IP	13550
		2235_2252>AAT	E746_T751>I	13551
		2235_2251>AATTC	E746_T751>IP	13552
		2253_2276del24	S752_I759del	13556
		2237_2257>TCT	E746_P753>VS	18427
		2238_2252del15	L747_T751del	23571
		2233_2247del15	K745_E749del	26038
Exon 20	S768I	2303 G>T	S768I	6241
	T790M	2369 C>T	T790M	6240
	Ex20Ins	2307_2308ins9GCCAGCGTG	V769_D770insASV	12376
		2319_2320insCAC	H773_V774insH	12377
		2310_2311insGGT	D770_N771insG	12378
		2311_2312ins9GCGTGGACA	D770_N771insSVD	13428
		2309_2310AC>CCAGCGTGGAT	V769_D770insASV	13558
Exon 21	L858R	2573 T>G	L858R	6224
		2573_2574 TG>GT	L858R	12429
	L861Q	2582 T>A	L861Q	6213

Non-clinical performance evaluation

Analytical performance

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

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 of healthy-donor K2 EDTA plasma EGFR wild-type specimens were evaluated. Using the analysis prescribed in the CLSI EP17-A2 guideline[7], the Limit of Blank was determined to be zero for all mutations.

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 4.

Table 4 Limit of detection of cobas® EGFR Test with K2 EDTA Plasma

EGFR Exon	EGFR Mutation Group	EGFR Nucleic Acid Sequence	Intact* DNA LOD (copies/mL)	Sheared** DNA LOD (copies/mL)	COSMIC ID[6]
18	G719A	2156G>C	100	100	6239
19	Ex19Del	2235_2249del15	25	75	6223
20	S768I	2303G>T	20	25	6240
20	T790M	2369C>T	25	100	6241
20	Ex20Ins	2307_2308ins9GCCAGCGTG	80	25	12376
21	L858R	2573T>G	10	100	6224
21	L861Q	2582T>A	30	30	6213

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 220bp, 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.

Cross reactivity to other Exon 18, 19, 20, and 21 mutations

The **cobas**® EGFR Test also picks up additional mutations not claimed in Table 3 which are shown below in Table 5. Analytical performance of the **cobas**® EGFR Test in detecting these mutations has not been evaluated.

Table 5 Additional Mutations observed with the cobas® EGFR Test

Exon	Mutation Sequence	AA Change	COSMIC ID[6]
19	2254_2277del24	S752_I759delSPKANKEI	6256
	2236_2256>ATC	E746_S752>I	133190
	2239_2256>CAG	L747_S752>Q	Not Found
	2239_2264>GCCAA	L747_A755>AN	85891
	2240_2264>CGAGAGA	L747_A755>SRD	Not Found

Specificity – microorganism

Specificity of the **cobas**®EGFR Test was evaluated by testing *Staphylococcus epidermidis* at 1 x 10^6 colony forming units, which was found not to cross react or interfere with the **cobas**®EGFR Test when added to healthy-donor K2 EDTA plasma samples containing wild-type and mutant EGFR sequences.

Interference

Triglycerides (37 mM. CLSI recommended high concentration[8]), 0.2 g/L of bilirubin (unconjugated or conjugated, CLSI recommended high concentration[8]), and hemoglobin (1.5 g/L) have been shown not to interfere with the **cobas**®EGFR Test when the potentially interfering substance was added to healthy-donor K2 EDTA plasma samples containing wild-type and mutant EGFR sequences. Hemoglobin at a concentration of 2.0. g/L in plasma has been shown to interfere with the **cobas**®EGFR Test. Albumin at a concentration of 60 g/L (60 g/L, CLSI recommended high concentration[8]), may interfere with the **cobas**®EGFR Test.

The study results demonstrate that EDTA, Neupogen, and TARCEVA® do not interfere with the performance of the **cobas**®EGFR Test when the potentially interfering substance was added to healthy-donor K2 EDTA plasma samples containing wild-type and mutant EGFR sequences.

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 6).

Table 6 cobas® EGFR Test vs. MiSeq sequencing

Measure of Agreement	Percent Agreement (n)	95% CI
Positive Percent Agreement (PPA)	80.0% (28/35)	64.1, 90.0%
Negative Percent Agreement (NPA)	94.9% (37/39)	83.1, 98.6%
Overall Percent Agreement (OPA)	87.8% (65/74)	78.5, 93.5%

Linearity

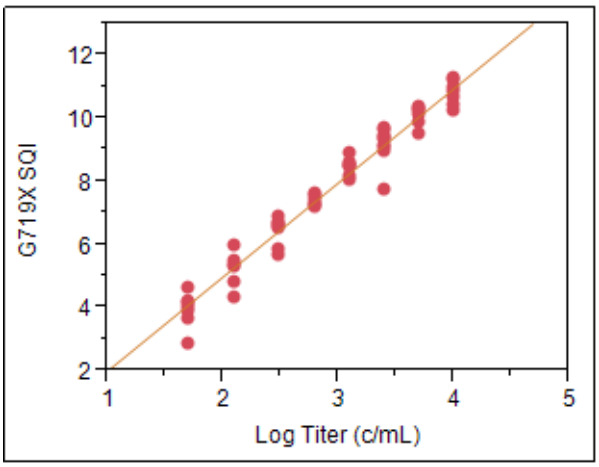
The linearity study of **cobas**® EGFR Test was performed with dilution series of at least 8 panel members spanning the linear range for the predominant mutation for each EGFR mutation class reported by the test. Panel members were prepared by diluting cell line DNAs containing each of predominant mutations into healthy-donor K2 EDTA plasma that is wild-type for EGFR. The evaluation was performed according to CLSI Guideline EP06-AE.[9] 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 7 and the corresponding graphs for one lot are shown in Figure 3 through Figure 9.

Table 7 Linear range of the cobas® EGFR Test with K2 EDTA Plasma

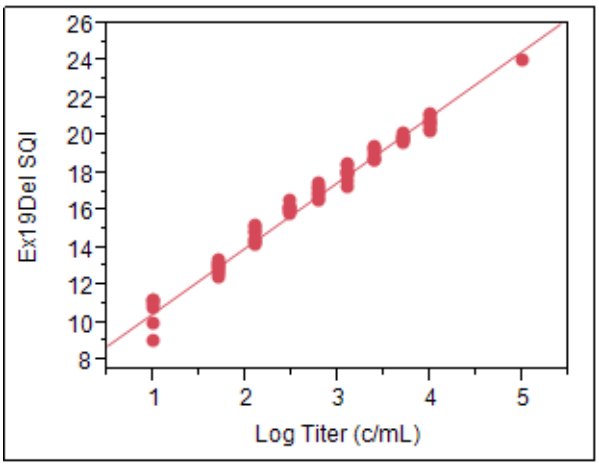
EGFR Exon	EGFR Mutation	Target Nucleic Acid Sequence	Linear Range (copies/mL)
18	G719A	2156 G>C	50 - 1E+04
19	Exon 19 Deletion	2235-2249del15	10 - 1E+05
20	S768I	2303 G>T	10 - 1E+05
20	T790M	2369 C>T	50 - 1E+05
20	Exon 20 Insertion	2307_2308ins9 (GCCAGCGTG)	10 - 1E+05
21	L858R	2573 T>G	10 - 1E+05
21	L861Q	2582 T>A	10 - 1E+05

Figure 3 Linearity of mutant DNA in K2 EDTA Plasma: G719A cell line DNA



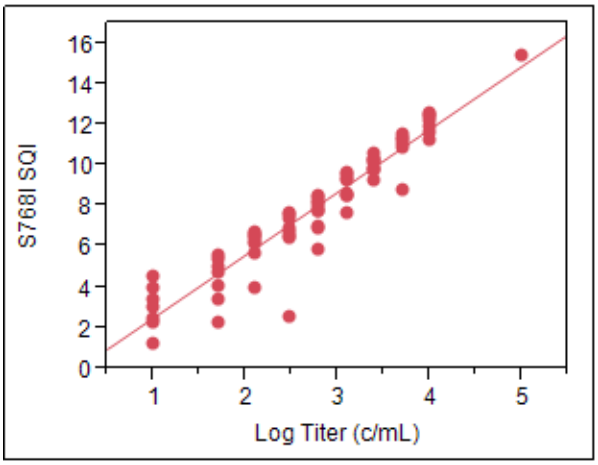
SQI = -0.987 + 2.986 * Log Copies per mL
$R^2 = 0.968$

Figure 4 Linearity of mutant DNA in K2 EDTA Plasma: Ex19 Del cell line DNA



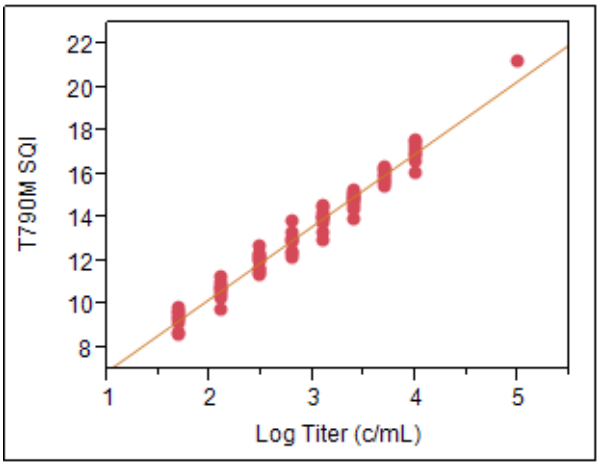
SQI = 7.042 + 3.507 * Log Copies per mL
$R^2 = 0.981$

Figure 5 Linearity of mutant DNA in K2 EDTA Plasma: S768I cell line DNA



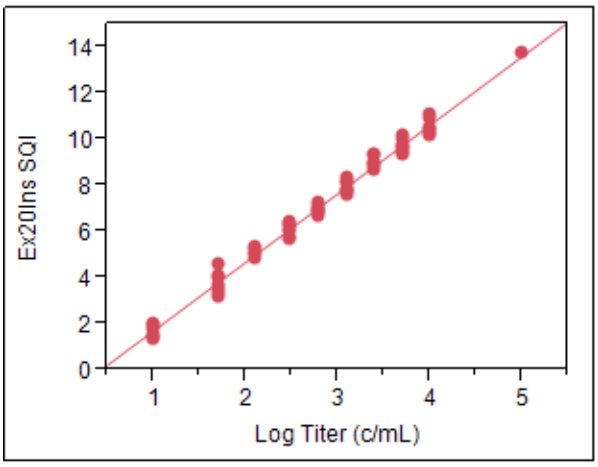
SQI = -0.578 + 3.093 *Log Copies per mL
$R^2 = 0.912$

Figure 6 Linearity of mutant DNA in K2 EDTA Plasma: T790M cell line DNA



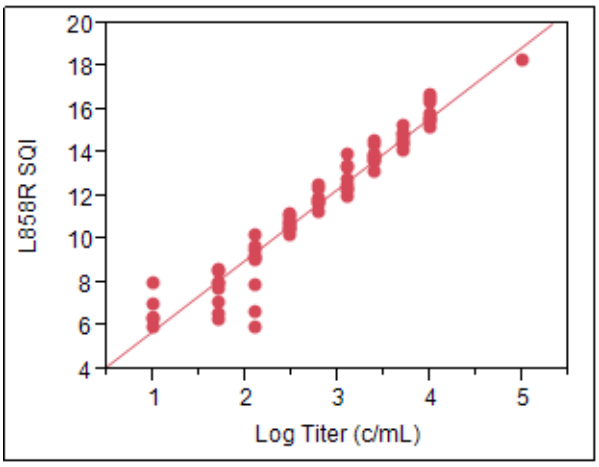
SQI = 3.593 + 3.352 *Log Copies per mL
$R^2 = 0.973$

Figure 7 Linearity of mutant DNA in K2 EDTA Plasma: Ex20Ins cell line DNA



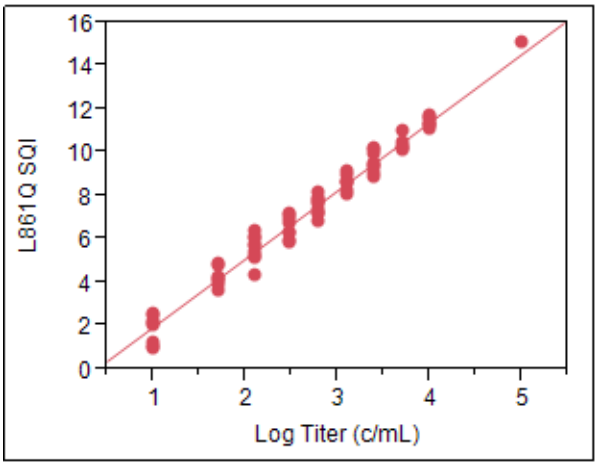
SQI = -1.268 + 2.973 *Log Copies per mL
R^2 = 0.990

Figure 8 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 9 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 a panel of twelve samples composed of 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 into eleven samples 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 twelve samples was tested in duplicate by two operators, using two different reagent lots over 4 days across two **cobas z** 480 analyzers (N=32 per sample). The **cobas**®EGFR Test had a correct call rate of 99.2% (381/384).

Table 8 lists the mean SQI and the SQI SD from the repeatability study. All 32 replicates for the *wild-type* sample yielded the expected 'No Mutation Detected' result.

Table 8 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	2156 G>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	2303 G>T	6.00E+01	5.99	0.45
			1.00E+03	8.49	0.43
			5.00E+04	14.13	0.43
20	T790M	2369 C>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_2308ins9 (GCCAGCGTG)	2.40E+02	4.92	0.43
			1.00E+03	6.77	0.40
			5.00E+04	12.61	0.60
21	L858R	2573 T>G	1.20E+02	9.81	0.47
			1.00E+03	12.91	0.28
			5.00E+04	17.21	0.81
21	L861Q	2582 T>A	4.50E+01	3.58	0.73
			1.00E+03	7.91	0.45
			5.00E+04	10.06	0.60
EGFR wild-type			0	NMD	NMD

"NMD" = No Mutation Detected

Additional information

Symbols

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

Table 9 Symbols used in labeling for Roche PCR products

	Ancillary Software		Lower Limit of Assigned Range
	Authorized Representative in the European community		Manufacturer
	Barcode Data Sheet		Store in the dark
	Batch code		Contains Sufficient for <*n*> tests
	Biological Risks		Temperature Limit
	Catalogue number		Test Definition File
	Consult instructions for use		Upper Limit of Assigned Range
	Contents of kit		Use-by date
	Distributed by		Global Trade Item Number

US Customer Technical Support 1-800-526-1247

Manufacturer and distributors

Table 10 Manufacturer and distributors

Manufactured in the United States
Roche Diagnostics GmbH
Sandhofer Strasse 116
68305 Mannheim, Germany
www.roche.com

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

Roche Diagnostics GmbH
Sandhofer Strasse 116
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Document revision

Document Revision Information	
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.
Doc Rev. 3.0 01/2017	Updated **Principles of the procedure** in the Summary and explanation of the test. Updated **Non-clinical performance evaluation** section: clarified repeatability results; included limit of blank, cross-reactivity, microorganism specificity, and interference test results. Clarified storage conditions for processed samples and plasma and specimens. Clarified **Reagent storage and handling** for **cobas**® cfDNA Sample Preparation Kit. Updated safety symbols and warnings, including instructions for Spillage and cleaning. Updated **References** section. Revised document content for consistency between product versions. Please contact your local Roche Representative if you have any questions.