FOR LIFE SCIENCE RESEARCH ONLY. NOT FOR USE IN DIAGNOSTICS PROCEDURES.

KRAS Mutation Test v2 (LSR)

24 reactions

P/N: 07989270001

Version 04

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KRAS Mutation Test v2 (LSR): Intended Use

The **KRAS Mutation Test v2 (LSR)** is an allele-specific, real-time PCR test for the qualitative detection and identification of exon 2, 3, and 4 mutations in the V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) gene from formalin-fixed, paraffin-embedded tissue (FFPET) and from cfDNA derived from plasma. It is intended for life science research only and is not for use in diagnostic procedures.

Summary and explanation of the test

Principles of the procedure

The **KRAS Mutation Test v2 (LSR)** is based on two processes: (1) manual sample preparation to obtain genomic 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 test is designed to detect 28 unique mutations at a percent mutation of 1% or greater, unless otherwise indicated. The list of mutations is shown below in **Table 1**.

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.

Table 1 The KRAS Mutation Test (LSR) is designed to detect the following mutations

Exon	KRAS Mutation Group	KRAS Mutation	Nucleotide Nomenclature	Protein Nomenclature	COSMIC ID
Exon 2	G12X *	G12A	c.35G>C	p.Gly12Ala	522
		G12C*	c.34G>T	p.Gly12Cys	516
		G12D	c.35G>A	p.Gly12Asp	521
		G12R	c.34G>C	p.Gly12Arg	518
		G12S	c.34G>A	p.Gly12Ser	517
		G12V	c.35G>T	p.Gly12Val	520
	G13X	G13A	c.38G>C	p.Gly13Ala	533
		G13C	c.37G>T	p.Gly13Cys	527
		G13D	c.38G>A	p.Gly13Asp	532
		G13R	c.37G>C	p.Gly13Arg	529
		G13S	c.37G>A	p.Gly13Ser	528
		G13V	c.38G>T	p.Gly13Val	534
Exon 3	A59X	A59E	c.176C>A	p.Ala59Glu	547
		A59G	c.176C>G	p.Ala59Gly	28518
		A59S	c.175G>T	p.Ala59Ser	1235389
		A59T	c.175G>A	p.Ala59Thr	546
	Q61X	Q61E	c.181C>G	p.Gln61Glu	550
		Q61Hc	c.183A>C	p.Gln61His	554
		Q61Ht	c.183A>T	p.Gln61His	555
		Q61K	c.181C>A	p.Gln61Lys	549
		Q61L	c.182A>T	p.Gln61Leu	553
		Q61P	c.182A>C	p.Gln61Pro	551
		Q61R	c.182A>G	p.Gln61Arg	552
Exon 4	K117X	K117Nc	c.351A>C	p.Lys117Asn	19940
		K117Nt	c.351A>T	p.Lys117Asn	28519
	A146X	A146P	c.436G>C	p.Ala146Pro	19905
		A146T	c.436G>A	p.Ala146Thr	19404
		A146V	c.437C>T	p.Ala146Val	19900

*For the FFPET sample type only, G12C is reported separately from other mutations in G12X. For the plasma sample type, G12C is reported as G12X.

Reference Sequence

Please refer to the following sources for reference sequences for KRAS.

KRAS: LRG_344t1

PCR amplification

Target selection

The **KRAS Mutation Test v2 (LSR)** uses primers that define specific base-pair sequences for each of the targeted mutations. Amplification occurs only in the regions of the KRAS gene between the primers; the entire gene is not amplified. An endogenous Internal Control (IC) is amplified in each sample to verify reagent and DNA quality. The IC detects a region at the 3' end of the KRAS gene that does not typically experience somatic mutations. The targeted KRAS sequences range from 79 – 114 base pairs.

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 KRAS 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 **KRAS Mutation Test v2 (LSR)** 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 targeted sequences is 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 **KRAS Mutation Test v2 (LSR)** kit by the use of AmpErase (uracil-N-glycosylase) enzyme and deoxyuridine triphosphate (dUTP). 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.

Sample preparation

Note: ***The cobas® DNA Sample Preparation Kit (P/N: 05985536190, for FFPET) or cobas® cfDNA Sample Preparation Kit (P/N: 07247737190, for plasma) is strongly preferred for use with the KRAS Mutation Test v2 (LSR) kit, though it is not required. Alternative sample preparation methods should be validated for use with the KRAS Mutation Test v2 (LSR).***

Note: **The information below applies only to the cobas® DNA Sample Preparation Kit and cobas® cfDNA Sample Preparation Kit (P/N: 05985536190 and P/N: 07247737190, respectively).**

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 µm section of an FFPET sample is lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released genomic DNA from DNases.

Plasma samples are processed and circulating cell free DNA (cfDNA) isolated using the **cobas®** cfDNA Sample Preparation Kit 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 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 determined using a spectrophotometer 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 **KRAS Mutation Test v2 (LSR)** kit.

Materials and reagents

Materials and reagents provided

Table 2 Contents of the KRAS Mutation Test v2 (LSR) Kit

Kit	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning
KRAS Mutation Test v2 (LSR) 24 Tests (P/N: 07989270001)	**MMX-1** **(**KRAS v2 Master Mix 1) White Cap	2 x 0.48 mL	N/A
	MMX-2 (KRAS v2 Master Mix 2) Brown Cap	2 x 0.48 mL	N/A
	MMX-3 **(**KRAS v2 Master Mix 3) Blue Cap	2 x 0.48 mL	N/A
	MgAc (Magnesium acetate) Yellow Cap	2 x 0.6 mL	N/A
	KRAS MC (KRAS Mutant Control) Red Cap	2 x 0.4 mL	N/A
	DNA SPEC DIL (DNA Specimen Diluent; SD)	2 x 3.5 mL	N/A

Warnings and suggested handling requirements

Note: Safety Data Sheets are available upon request from your local Roche office.

1. **FOR LIFE SCIENCE RESEARCH ONLY. Not for use in diagnostic procedures.**
2. Do not pipette by mouth.
3. Do not eat, drink or smoke in laboratory work areas.
4. 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.
5. Hands must be washed thoroughly and gloves must be changed between handling samples and reagents to prevent contamination. Avoid microbial and DNA contamination of reagents.
6. To avoid contamination of the working Master Mix (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.
7. Dispose of unused reagents and waste in accordance with country, federal, state and local regulations. Do not use kits after their expiration dates. Do not pool reagents from different kits or lots.
8. All disposable items are for one time use. Do not reuse.
9. 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.All disposable items are for one time use. Do not reuse.
10. Do not use sodium hypochlorite solution (bleach) for cleaning the **cobas z** 480 analyzer. Clean the **cobas z** 480 analyzer according to procedures described in the **cobas z** 480 analyzer Instrument Manual.
11. 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.
12. The use of sterile disposable pipettes and DNase-free pipettor tips is recommended.
13. **Specific to the cobas® DNA Sample Preparation Kit (P/N: 05985536190) and the cobas® cfDNA Sample Preparation Kit (P/N: 07247737190):**
    a. **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 z** 480 analyzer, follow the instructions in the **cobas z** 480 analyzer Instrument Manual.
    b. 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.
    c. Samples should be handled as infectious using safe laboratory procedures such as those outlined in Biosafety in Microbiological and Biomedical Laboratories and in the CLSI Document M29-A3.
    d. **DNA PBB** and **DNA TLB** contain a non-ionic detergent which is an irritant to mucous membranes. Avoid contact with eyes, skin, and mucous membranes.
14. **DNA TLB**, **DNA EB**, **MgAc**, **MMX-1**, **MMX-2**, **MMX-3**, **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.

Storage requirements

1. **cobas® DNA Sample Preparation Kit (P/N: 05985536190) and cobas® cfDNA Sample Preparation Kit (P/N: 07247737190):**
   a. With the exception of the PK reagent, do not freeze reagents.
   b. Store **DNA TLB**, **DNA PBB**, **WB I**, **WB II**, **DNA EB**, **PK**, **FT**, and **CT** at 15ºC to 30ºC. Once opened, **DNA TLB**, **DNA PBB**, **WB I**, **WB II**, **DNA EB**, and **PK** are stable for up to 8 uses over 90 days or until the expiration date, whichever comes first.
   c. 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.
   d. After addition of absolute ethanol, store **WB I** and **WB II** at 15ºC to 30ºC. These working solutions are stable for 8 uses over 90 days or until the expiration date, whichever comes first.
2. **KRAS Mutation Test v2 (LSR) (P/N: 07989270001)**
   a. Store **MMX-1**, **MMX-2**, **MMX-3**, **MgAc**, **MC**, and **SD** at 2ºC to 8°C.
   b. **MMX-1**, **MMX-2**, **MMX-3**, and working MMX (prepared by the addition of **MgAc** to **MMX-1** or **MMX-2** or **MMX-3**) should be protected from prolonged exposure to light.
   c. Processed samples (extracted DNA) are stable for up to 24 hours at 15ºC to 30°C or up to 14 days at 2ºC to 8°C or up to 60 days at -15ºC to -25°C or after undergoing 3 freeze thaws when stored at -15ºC to -25°C.

Additional materials required

Table 3 Materials Needed but Not Provided

Materials	P/N
Portable media drive (*e.g.*, flash drive)	Any vendor
Xylene (ACS, > 98.5% xylenes) [for FFPET only]	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 HycloneTM 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
Table top centrifuge* (capable of 6,000 x g while holding 50mL conical tubes in a swing-bucket rotor) [for Plasma only]	Eppendorf model 5810 or equivalent
Two dry heat blocks capable of heating microcentrifuge tubes to 56ºC and 90ºC* [for FFPET only]	Any vendor
Locking-lid microcentrifuge tubes (1.5mL, sterile, RNase/ DNase free, PCR grade)	Eppendorf 022363204 or equivalent
Microcentrifuge tube racks	Any vendor
15-mL Sterile conical plastic tubes [for Plasma only]	Any vendor
Spectrophotometer for measuring DNA concentration* [for FFPET only]	Any vendor
Vortex mixer*	Any vendor
Disposable gloves, powder-free	Any vendor
Calibrated thermometers for dry heat block* [for FFPET only]	Any vendor
Waterbath* capable of maintaining 37ºC	Any vendor
Single edged blade or similar [for FFPET only]	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 User Defined Workflow (UDF)

Test procedure

Running the test

Table 4 Workflow Steps: KRAS Mutation Test v2 (LSR)

1	Start the system
2	Perform instrument maintenance
3	Remove samples and reagents from storage
4	Deparaffinize samples [for FFPET only]
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	Enter the run and specimen information in the UDF software and start the run
14	Export raw data file when PCR reaction is complete
15	Upload data file to Data Analysis Portal on Oncology Life Science Research Kits Website
16	Review results

Sample collection, transport, and storage

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

Sample collection

FFPET and plasma samples may be used with the **KRAS Mutation Test v2 (LSR)**.

Plasma should be separated from blood within 4 hours of collection and stored as indicated below until tested.

The **cobas**® DNA Sample Preparation Kit (P/N: 05985536190) has been developed for use with FFPET samples. The **cobas**® cfDNA Sample Preparation Kit (P/N: 07247737190) has been developed for use with EDTA anti-coagulated plasma samples.

Sample transport, storage, and stability

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

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.

Plasma samples 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	24 hours

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

Instructions for use

Note: For FFPET samples, only sections of 5 µm thickness containing at least 10% tumor content by area are to be used in the KRAS Mutation Test v2 (LSR). Any sample containing less than 10% tumor content by area should be macro-dissected following deparaffinization.

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 test kits will be required.

The **KRAS Mutation Test v2 (LSR)** contains sufficient reagents for 8 runs of 3 samples (plus controls) for a maximum of 24 samples per kit.

Workflow

The **KRAS Mutation Test v2 (LSR)** consists of sample preparation (using the **cobas**® DNA Sample Preparation Kit or cobas® cfDNA Sample Preparation Kit or another compatible method) followed by amplification/detection on the **cobas z** 480 analyzer using the **KRAS Mutation Test v2 (LSR)**.

Note: If using a DNA isolation method other than the cobas® DNA Sample Preparation Kit (P/N: 05985536190) or the cobas® cfDNA Sample Preparation Kit (P/N: 07247737190), please proceed to the Amplification and detection section.

Table 5 Reagent Preparation (if using the cobas® DNA Sample Preparation Kit)

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 locking lid microcentrifuge tubes and store at -20ºC 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 is only for FFPET; 250 µL is required for each plasma 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.

SECTION A: SAMPLE PREPARATION FOR FFPET SAMPLES

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 macrodissected.

1. Add a slide with a mounted 5 µm 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 locking-lid microcentrifuge tube for each sample with the sample identification information.
6. Add 180 µL **DNA TLB** to the 1.5-mL locking-lid microcentrifuge tube.
7. Add 70 µL of reconstituted PK to the locking-lid microcentrifuge tube containing **DNA TLB**.
8. Scrape the tissue off the slide and into the locking-lid 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 µm FFPET section into a 1.5 mL locking-lid microcentrifuge tube labeled with the sample identification information for each sample.
2. Add 500 µL Xylene to the locking-lid 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 locking-lid microcentrifuge tube labeled as NC. 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 (**NC**) mixture 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 locking-lid microcentrifuge tube).

Note: During the incubation, label the required number of elution tubes (1.5 mL locking-lid 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 locking-lid 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 (NC) does not need to be measured.

3. The DNA Stock concentration from the samples must be > 2 ng/µL to perform the **KRAS Mutation Test v2 (LSR)**. 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 KRAS Mutation Test v2 (LSR). 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, acquire another FFPET sample section.

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.

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 microcentrifuge tubes for DNA samples and Negative Control (**NC**) dilutions and label them in the Sample Addition Area.
2. Using a pipettor with an aerosol-resistant pipette tip, pipette 45 µL of Specimen Diluent into the 1.5 mL microcentrifuge tube labeled as the **NC**.
3. Using a pipettor with an aerosol-resistant pipette tip, pipette the calculated volume of **SD** into each labeled sample microcentrifuge tube.
4. Vortex each sample DNA stock and Negative Control for 10 seconds.
5. Using a pipettor with an aerosol-resistant pipette tip, gently pipette 45µL of the processed Negative Control into the 1.5 mL microcentrifuge tube labeled as the **NC**.
6. Using a pipettor with an aerosol-resistant pipette tip, gently pipette the calculated volume of each sample DNA stock into the corresponding, labeled sample tube containing **DNA SD**.
   Use a new pipette tip for each sample.
7. Cap and mix each diluted DNA sample and **NC** by vortexing 10 seconds.
8. Change gloves.

SECTION B: SAMPLE PREPARATION FOR PLASMA SAMPLES

cfDNA 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.

Note: Ensure that the PK is added to the plasma first, then add the DNA PBB.

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 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 in each **CT** into chemical waste and properly dispose of old **CT**.
18. Add 500 µL working **WB II** to each **FT**.

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

19. Centrifuge the **FT**/**CT** units at 8,000 x *g* for 1 minute.
20. 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**.
21. Centrifuge the **FT**/**CT** units at 16,000 to 20,000 x *g* for 1 minute to dry the filter membranes.
22. Place each **FT** into 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 used **CT**.

23. Add 100 µL **DNA EB** to the center of each **FT** membrane without touching the **FT** membrane.
24. Incubate the **FT** with elution tube at 15°C to 30°C for 5 minutes.
25. 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.
26. Discard the **FT**. Close the caps on the elution tubes.

Note: Pipetting from the bottom of the elution tube may disrupt the pellet and adversely affect test results.

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. Slowly pipette from the top half of the eluate.

FOR BOTH FFPET AND PLASMA SAMPLES

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.***

Test order set-up

Generate a plate map with the position of all the samples and controls in the run. The MC is loaded into positions A01 – A03 on the plate. The NC 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 1**.

Figure 1 Plate layout for the KRAS Mutation Test v2 (LSR)

	1	2	3	4	5	6	7	8	9	10	11	12
A	MC MMx1	MC MMx2	MC MMx3	Spcmn7 MMx1	Spcmn7 MMx2	Spcmn7 MMx3	Spcmn15 MMx1	Spcmn15 MMx2	Spcmn15 MMx3	Spcmn23 MMx1	Spcmn23 MMx2	Spcmn23 MMx3
B	NC MMx1	NC MMx2	NC MMx3	Spcmn8 MMx1	Spcmn8 MMx2	Spcmn8 MMx3	Spcmn16 MMx1	Spcmn16 MMx2	Spcmn16 MMx3	Spcmn24 MMx1	Spcmn24 MMx2	Spcmn24 MMx3
C	Spcmn1 MMx1	Spcmn1 MMx2	Spcmn1 MMx3	Spcmn9 MMx1	Spcmn9 MMx2	Spcmn9 MMx3	Spcmn17 MMx1	Spcmn17 MMx2	Spcmn17 MMx3	Spcmn25 MMx1	Spcmn25 MMx2	Spcmn25 MMx3
D	Spcmn2 MMx1	Spcmn2 MMx2	Spcmn2 MMx3	Spcmn10 MMx1	Spcmn10 MMx2	Spcmn10 MMx3	Spcmn18 MMx1	Spcmn18 MMx2	Spcmn18 MMx3	Spcmn26 MMx1	Spcmn26 MMx2	Spcmn26 MMx3
E	Spcmn3 MMx1	Spcmn3 MMx2	Spcmn3 MMx3	Spcmn11 MMx1	Spcmn11 MMx2	Spcmn11 MMx3	Spcmn19 MMx1	Spcmn19 MMx2	Spcmn19 MMx3	Spcmn27 MMx1	Spcmn27 MMx2	Spcmn27 MMx3
F	Spcmn4 MMx1	Spcmn4 MMx2	Spcmn4 MMx3	Spcmn12 MMx1	Spcmn12 MMx2	Spcmn12 MMx3	Spcmn20 MMx1	Spcmn20 MMx2	Spcmn20 MMx3	Spcmn28 MMx1	Spcmn28 MMx2	Spcmn28 MMx3
G	Spcmn5 MMx1	Spcmn5 MMx2	Spcmn5 MMx3	Spcmn13 MMx1	Spcmn13 MMx2	Spcmn13 MMx3	Spcmn21 MMx1	Spcmn21 MMx2	Spcmn21 MMx3	Spcmn29 MMx1	Spcmn29 MMx2	Spcmn29 MMx3
H	Spcmn6 MMx1	Spcmn6 MMx2	Spcmn6 MMx3	Spcmn14 MMx1	Spcmn14 MMx2	Spcmn14 MMx3	Spcmn22 MMx1	Spcmn22 MMx2	Spcmn22 MMx3	Spcmn30 MMx1	Spcmn30 MMx2	Spcmn30 MMx3

Note: ***Where MC = Mutant Control, NC = Negative Control, Spcmn# = Sample ID, and MMx# corresponds to Master Mix 1,2, or 3.***

Note: ***Any given sample must be added to three consecutive columns in one row in order to generate a response.***

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

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

Reaction set-up

Preparation of working master mixes (MMX-1, MMX-2 and MMX-3)

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

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

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

Prepare three bulk working MMX, one containing **MMX-1**, one containing **MMX-2**, and the other containing **MMX-3** in separate 1.5 mL locking-lid microcentrifuge tubes.

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

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

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

   Volume of **MgAc** required = (Number of Samples + 2 Controls +1) x 6.5 µL

Use **Table 6** 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 6 Volumes of reagents needed for working MMX-1, working MMX-2 and working MMX-3

		# of Samples*									
		1	**2**	**3**	**6**	**9**	**12**	**15**	**18**	**21**	**24**
MMX	**20 µL**	80	100	120	180	240	300	360	420	480	540
MgAc	**7 µL**	28	35	42	63	84	105	126	147	168	189
Total Vol. for Each Working MMX (µL)		**108**	**135**	**162**	**243**	**324**	**405**	**486**	**567**	**648**	**729**

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

3. Remove the appropriate number of **MMX-1**, **MMX-2**, **MMX-3**, and **MgAc** vials from 2°C to 8°C storage. Vortex each reagent for 5 seconds and collect liquid at the bottom of the tube before use. Label a sterile microcentrifuge tube for working MMX-1, working MMX-2, and working MMX-3.
4. Add the calculated volume of **MMX-1** or **MMX-2** or **MMX-3** to their respective working MMX tube.
5. Add the calculated volume of **MgAc** to the working MMX tubes.
6. Vortex the tubes for 3 to 5 seconds to ensure adequate mixing.

Note: Samples and controls should be added to the 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 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 **MMX-1**) to the AD-plate wells in columns 01, 04, 07, and 10, as needed.
   - Add working MMX-2 (containing **MMX-2**) to the AD-plate wells in columns 02, 05, 08, and 11, as needed.
   - Add working MMX-3 (containing **MMX-3**) to the AD-plate wells in columns 03, 06, 09, and 12, as needed.
2. Pipette 25 µL of **MC** into wells **A01**, **A02**, and **A03** of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.
3. Using a new pipettor tip, pipette 25 µL of Negative Control (**NC**) into wells **B01**, **B02**, and **B03** of the AD-plate; mix well using pipette to aspirate and dispense within the well a minimum of two times.

Note: Each run must contain Mutant Control (MC) in wells A01, A02 and A03, and Negative Control (NC) in wells B01, B02, and B03 or the run will be invalidated by the cobas z 480 analyzer.

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

4. Using new pipettor tips for each diluted sample DNA, add 25 µL of the first sample DNA to wells C01, C02, and C03 of the AD-plate, using a new tip for the addition of the sample DNA to each well; mix each well using a pipette to aspirate and dispense within the well a minimum of two times. Repeat this procedure for the DNA from each sample and follow the template in Figure 1 until all samples' DNA dilutions are loaded onto the 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 AD-plate with sealing film (supplied with the plates). Use the sealing film applicator to seal the film firmly to the AD-plate.
6. Confirm that all liquid is collected at the bottom of each well before starting PCR.

Note: Amplification and detection should be started within 1 hour after the addition of the first sample DNA dilution to the working MMX.

Instrument setup and data analysis using the cobas z 480 analyzer

Note: Please see the cobas® 4800 System Operator's Manual User Defined Workflow (UDF) (Software Version 1.0 or 2.0) for more information on the UDF workflow and subset creation.

Note: Portable external storage media may contain viruses or other malware software which could affect the correct operation of the system and/or provide unauthorized access to it. Users are advised to perform a virus scan on any external portable media with an up-to-date anti-virus software before using it in the system.

Importing the run template file

1. On a personal computer, open Internet Browser.

Note: ***Different browsers may vary in performance. Please check the Data Analysis tab at http://oncologyresearchkits.roche.com for a list of current supported browsers.***

2. Visit http://oncologyresearchkits.roche.com
3. Within the Data Analysis tab, download the **AS-PCR_Profile_96w.ixo** file to the computer and transfer to portable media unit (*e.g.*, USB drive).
4. Turn on the **cobas z** 480 analyzer if it is turned off. Allow system to fully initialize before starting the run.
5. Turn on/reboot the **cobas**® 4800 workstation and select the UDF partition.
6. Double click on the **cobas**® 4800 system User Defined Workflow (UDF) software icon and log on to perform the run using the specified lab user ID and password (Operator level).
7. Insert flash drive or other portable media unit from Step 3 into the **cobas z** 4800 workstation.
8. Go to Window and choose NAVIGATOR and IMPORT. Go to .ixo file location and click on **AS-PCR_Profile_96w.ixo**.
9. Click OPEN and then click the SAVE icon.
10. Under the Root Folder go to the Templates folder.
11. In the Templates folder go to the Run Templates folder and click on **AS-PCR_Profile_96w.ixo** file and then click on the CHECK box.

Performing the run

Note: ***Users will be required to manually enter IDs for all samples tested. Plate wells used must be included in a subset labeled KRAS v2 FFPET (exact name is required) or KRAS v2 Plasma (exact name is required).***

1. Turn on the **cobas z** 480 analyzer if it is turned off. Allow system to fully initialize before starting the run.
2. If necessary, reboot the **cobas**® 4800 workstation and log into the User-Defined Workflow (UDF) partition on the system.
3. Double click on the **cobas**® 4800 system User-Defined Workflow (UDF) software icon and log on to per form the run using the specified lab user ID and password (Operator level).
4. Press the load button on **cobas z** 480 analyzer and load the 96-well plate into the instrument with the short plate edge with the beveled corner pointing away from the instrument.
5. Press the load button on **cobas z** 480 analyzer to close the loader.
6. In the “Window:” dropdown, select the Overview window, and then click on the “New Experiment from Template” button.
7. Select the **AS-PCR_Profile_96w.ixo** profile from the Run Templates window and click the check button.
8. Click on the Sample Editor and enter the sample names different from default name for the wells being used. Click on Subset Editor, then click the + button. Under “New Subset 1”, select the wells being used and under “Subsets”, change the subset name “New Subset 1” to **KRAS v2 FFPET or KRAS v2 Plasma**.
9. Click on Experiment button on the left side of the screen, then click the “Start Run” button.
10. Save the run by typing the experiment name in the “Name” section. Then click on the check button.

Note: ***Each plate must contain both a Mutant Control (MC) in positions A01 – A03 and a Negative Control (NC) in positions B01 – B03. However, if users choose to run more than one test parameter on the plate (e.g., KRAS v2 FFPET and BRAF/NRAS FFPET), a new row of test-specific Mutant Controls and Negative Controls must be included at the top of a new column, with the Mutant Controls in the first row and the Negative Controls in the second.***

Data analysis

Note: ***It is NOT recommended to use Cp values generated by the cobas z 480 analyzer software to determine the presence of various mutations. Please use the values generated by the web-based data analysis only.***

Note: ***Data analyzed by the web analysis tool will only be available for 24 hours before being removed from the server. No data is stored permanently on any server, so if data is not retrieved within the 24-hour period, the .ixo file will need to be re-uploaded for analysis.***

Note: ***The Oncology Research Kit data analysis portal utilizes secure algorithms to automatically interpret data generated from Oncology LSR test kits. In order to avoid any concerns around data privacy, we prevent sample identification by requiring only a unique ID be used for each sample.***

1. The **cobas**® 4800 User Defined Workflow (UDF) software will automatically save all raw data files from completed runs onto the system.
2. Go to the Navigator window and in the Experiments folder locate the raw data file(s) you wish to analyze. (Raw data files are saved as Object files (*.ixo)).
3. Transfer the .ixo file(s) you wish to analyze to a portable media unit (*e.g.*, USB drive).
4. On personal computer, open internet browser.
5. Visit http://oncologyresearchkits.roche.com/data-analysis.
6. Enter login credentials OR register as a new Roche Life Science user.
7. Click the **Browse File** button and select the .ixo file you wish to analyze.
8. Click the **radio** button to select the **KRAS v2 FFPET or KRASv2 Plasma** test.
9. Click the **Analyze** button.
10. The file(s) will be available for download momentarily under RECENT HISTORY. Please click Check for Status to check if the result files are ready for download. Once ready, the files will be ready for download as a zip file containing a .pdf and .csv file.

Results

Interpretation of results

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 7**.

Table 7 Result interpretation for the KRAS Mutation Test v2 (LSR)

Test Result	Mutation Result	Interpretation
Mutation Detected	**G12X**	G12A, G12C**, G12D, G12R, G12S, G12V (G12C: plasma samples only)
	G12C	G12C** (FFPET samples only)
	G13X	G13A, G13C, G13D, G13R, G13S, G13V
	A59X	A59E, A59G, A59S, A59T
	Q61X	Q61E, Q61Hc, Q61Ht, Q61K, Q61L, Q61P, Q61R
	K117X	K117Nc, K117Nt
	A146X	A146P, A146T, A146V
No Mutation Detected (NMD)*	**N/A**	Mutation not detected in targeted KRAS regions.
Invalid	**N/A**	Sample result is invalid. Repeat the testing of samples with invalid results. For a list of result flags including flag descriptions, refer to **Table 8**.
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 KRAS regions because results depend on percent mutant sequences, adequate sample integrity, absence of inhibitors, and sufficient DNA to be detected.
** For the FFPET sample type only, G12C is reported separately from other mutations in G12X. For the plasma sample type, G12C is reported as G12X.

Note: Plasma testing will include an additional Semi Quantitative Index (SQI) value.

Semi quantitative index (SQI) for plasma samples

Note: The SQI will be heavily influenced by the pre-analytical methods employed using the KRAS Mutation Test v2 (LSR). For optimum results, cfDNA isolation using the cobas® cfDNA Sample Preparation Kit is highly recommended.

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.

List of error flags

Table 8 List of Error Flags

Flag Code	Description	Recommended Action
R25	Unexpected Number of Cycles	The run template may have an error or the run was aborted before the PCR thermal profile completed. Download and install the run template again to ensure correct values. Repeat the run.
R700, R706, R718, R724, R730, R736, R742, R748, R754, R760, R766	Mutant Control could not be detected.	Repeat the run. These flag codes indicate that the elbow determination algorithm encountered an error. This may occur in the event of an atypical or noisy fluorescence pattern.
R701, R707, R719, R725, R731, R737, R743, R749, R755, R761, R767	Mutant Control could not be detected.	Repeat the run. These flag codes indicate that a negative result occurred for the Mutant Control (*i.e.* Mutant Control DNA may have not been added to one or more wells).
R702, R708, R720, R726, R732, R738, R744, R750, R756, R762, R768	Mutant Control is out of range.	Repeat the run. These flag codes indicate that an observed elbow value for the Mutant Control was above the established threshold (*i.e.* elbow too high). This may occur in the event of 1) Incorrect preparation of working Master Mix, 2) Pipetting error when adding working Master Mix into a reaction well of the microwell plate, or 3) Pipetting error when adding Mutant Control into a reaction well of the microwell plate.
R703, R709, R721, R727, R733, R739, R745, R751, R757, R763, R769	Mutant Control is out of range.	Repeat the run. These flag codes indicate that an observed elbow value for the Mutant Control was below the established threshold (*i.e.* elbow too low). This may occur in the event of DNA contamination.
R772, R774, R778, R780, R782, R784, R786, R788, R790, R792, R794	Negative Control could not be detected.	Repeat the run. These flag codes indicate that the elbow determination algorithm encountered an error. This may occur in the event of an atypical or noisy fluorescence pattern.
R773, R775, R779, R781, R783, R785, R787, R789, R791, R793, R795	Negative Control is out of range.	Repeat the run. These flag codes indicate a positive result occurred for the Negative Control (*i.e.* a contamination event occurred).
R796, R801, R816, R821, R826, R836, R841, R846	No target could be detected.	Repeat the sample. These flag codes indicate the elbow determination algorithm encountered an error. This may occur in the event of an atypical or noisy fluorescence pattern.
R799, R800, R804, R805, R819, R820, R824, R825, R829, R830, R839, R840, R844, R845, R849, R850	Result is out of range.	Repeat the sample. These flag codes indicate either 1) An atypically low elbow value was observed for the sample, or 2) An atypical relationship between the Mutant elbow value and the Internal Control elbow value was observed for the sample.

Flag Code	Description	Recommended Action
R813, R833, R853	Internal Control out of range.	Repeat the sample. These flag codes indicate that the Internal Control result for the sample was not valid. The absence of a valid Internal Control result suggests: 1) Poor quality genomic DNA from the sample 2) Inadequate sample processing 3) The presence of PCR inhibitors in the sample 4) Rare mutations within the regions of the Genomic DNA covered by the Internal Control primers and/or probes 5) Sample DNA may have not been added to one or more wells 6) Other factors.
R814, R834, R854	Internal Control out of range.	Repeat the sample. These flag codes indicate an atypically low Internal Control elbow value was observed for the sample. This may occur if the PCR mixture is significantly overloaded with concentrated genomic DNA.
R811, R812, R831, R832, R851, R852	Internal Control could not be detected.	Repeat the sample. These flag codes indicate that the Internal Control result for the sample was not valid. The absence of a valid Internal Control result is suggestive of 1) Poor quality genomic DNA from the sample, 2) Inadequate sample processing, 3) The presence of PCR inhibitors in the sample, 4) Rare mutations within the regions of the genomic DNA covered by the Internal Control primers and/or probes, 5) Sample DNA may have not been added to one or more wells, or 6) Other factors.
R865	Mutant Control ID out of order.	Check for correct positioning of the Mutant Control. Three consecutive wells in the same row must have the same sample name and not be the default name.
R866	Negative Control ID out of order.	Check for correct positioning of the Negative Control. Three consecutive wells in the same row must have the same sample name and not be the default name.
R867	Specimen ID out of order.	Check for correct positioning of the Specimens. Three consecutive wells in the same row must have the same sample name and not be the default name.
R856, R857, R858	Unexpected Mutant Control signal during amplification.	Retest the sample.
R859, R860, R861	Unexpected Negative Control signal during amplification.	Retest the sample.
R862, R863, R864	Unexpected Specimen signal during amplification.	Retest the sample.

Retesting of FFPET samples with invalid results

1. Repeat dilution of the invalid sample DNA stock starting from “Dilution Calculation of Sample DNA Stock” and “Sample Dilution” section.
2. After performing the DNA stock dilution to 2 ng/µL, continue with “Preparation of working master mix (MMX-1, MMX-2 and MMX-3)” in the Amplification and Detection section.

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, repeat the entire test procedure for that sample, starting with deparaffinization and DNA Isolation using a new 5 µm FFPET tumor section.***

Retesting of plasma 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.

Procedural limitations

1. As with any test procedure, good laboratory technique is essential to the proper performance of this assay. Due to the high analytical sensitivity of this test, care should be taken to keep reagents and amplification mixtures free of contamination.
2. The **KRAS Mutation Test v2 (LSR)** was tested using the **cobas**® DNA Sample Preparation Kit (P/N: 05985536190) and the **cobas**® cfDNA Sample Preparation Kit (P/N: 07247737190).
3. G12X and G13X double mutation might be called as G12X mutation only when a smaller amount of G13X is present in a larger background of G12X.
4. 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 and PCR inhibitors.
5. Reliable results are dependent on sample fixation, transport, storage and processing.
6. The addition of AmpErase enzyme into the **KRAS Mutation Test v2 (LSR)** Master Mixes 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. The **cobas z** 480 analyzer was used during testing of this product. DNA input amounts other than 50 ng (FFPET) or 25µL eluate (plasma) per reaction well are not recommended.
8. Use of the data analysis software requires raw data from the **cobas z** 480 analyzer.

Analytical performance evaluation

The following data demonstrate the analytical performance of the **KRAS Mutation Test v2 (LSR)**.

The data are not intended to demonstrate any clinical performance claims for the test.
The **KRAS Mutation Test v2 (LSR)** is not intended for diagnostic procedures.

Sensitivity using plasmid DNA blends

Plasmid DNA constructs were blended with wild-type DNA to prepare percent mutant DNA samples ranging from 1% to 5%. A total of 4 replicates for each of 3 plasmid blends (12 total mutations) each at 3 percentage levels were tested with a DNA input of 50 ng. All mutations tested had an analytical sensitivity of at least 1% mutant.

Table 9 Sensitivity for the KRAS Mutation Test v2 (LSR) using Plasmid DNA Blends

				% Mutant		
				5%	2.5%	1%
Gene-Exon	**Mutation**	**Nucleic Acid Sequence**	**Cosmic ID**	**Hit Rate**		
2	G12C	c.34G>T	516	100%	100%	100%
2	G12R	c.34G>C	518	100%	100%	100%
2	G12V	c.35G>T	520	100%	100%	100%
2	G12D	c.35G>A	521	100%	100%	100%
2	G13C	c.37G>T	527	100%	100%	100%
2	G13D	c.38G>A	532	100%	100%	100%
3	A59T	c.175G>A	546	100%	100%	100%
3	Q61L	c.182A>T	553	100%	100%	100%
3	Q61Hc	c.183A>C	554	100%	100%	100%
4	K117Nt	c.351A>T	28519	100%	100%	100%
4	A146T	c.436G>A	19404	100%	100%	100%
4	A146V	c.437C>T	19900	100%	100%	100%

Exclusivity

The analogous sequences in the RAS genes corresponding to the targeted portions in the KRAS genes were cloned into individual plasmids. Plasmids of KRAS pseudogene, HRAS, and NRAS for exons 2, 3, and 4 were tested at 10,000 copies/25 µL. The non-targeted mutations did not cross-react when amplified in the **KRAS Mutation Test v2 (LSR)** master mixes.

FFPET samples:

Sensitivity using FFPET sample blends

DNA isolated from CRC and NSCLC FFPET samples with KRAS mutations were blended with DNA isolated from KRAS wildtype CRC and NSCLC FFPET samples to achieve blends targeting 10%, 5%, 2.5%, and 1% mutation levels in 50 ng DNA as determined by Illumina® MiSeq sequencing. Ten replicates for each mutation level and each sample were tested. The limit of detection for each sample was determined by comparing the Ct values to the Ct specifications for each channel in each master mix. 100% detection rate was demonstrated for samples with 10%, 5%, 2.5%, and 1% mutant DNA in a background of WT genomic DNA, shown in **Table 10**.

Table 10 Detection of FFPET Sample DNA Blends by the KRAS Mutation Test v2 (LSR)

		% Mutation			
		10%	5%	2.5%	1%
Gene-Exon	**Mutation**	**Hit Rate**			
2	G12C	100%	100%	100%	100%
2	G12D	100%	100%	100%	100%
2	G12V	100%	100%	100%	100%
2	G13C	100%	100%	100%	100%
3	A59G	100%	100%	100%	100%
3	Q61L	100%	100%	100%	100%
3	Q61Hc	100%	100%	100%	100%
4	K117Nt	100%	100%	100%	100%
4	A146T	100%	100%	100%	100%

Correlation to reference method

Comparison testing of 299 FFPET samples (99 CRC and 200 NSCLC) using the **KRAS Mutation Test v2 (LSR)** kit and Illumina® MiSeq sequencing was performed to determine the overall agreement between methods.

Table 11 Correlation in the Method Comparison for the KRAS Mutation Test v2 (LSR)

Correlation		**MiSeq**	
		+	**-**
KRAS v2	**+**	122	0
	-	1	176

PPA = 99.2% (122/123)
NPA = 100% (176/176)
Overall Agreement = 99.7% (298/299)

Repeatability

Repeatability of the **KRAS Mutation Test v2 (LSR)** was assessed using three mutant FFPET samples and three wildtype FFPET samples (one each per master mix). The KRAS mutant samples had the following mutations – G12D, G13D, and G12C. Each sample was tested in duplicate by 3 operators using 2 instruments over 3 days. A total of 216 out of 216 replicates (72 replicates per operator (3) = 216) gave the expected results, thus demonstrating a correct call accuracy of 100%.

Plasma samples:

Sensitivity using cfDNA from plasma samples

Two milliliters (2 mL) of normal human plasma specimens were spiked with sheared cell line DNA blends targeting 150, 100, 75, 50, and 25 copies mutant per mL plasma. 20 replicates for each sheared cell line at each input in normal cfDNA were tested. Mutations tested were: G12C, G12D, G12V, G13D, A59T, Q61Hc, Q61L, K117Nc, A146T. Specimen mutation percentage and copy number were confirmed by MiSeq. Subsequently, higher levels of WT sheared cell line DNA were spiked into normal human plasma and low copy number mutations were detectable in 64,000 copies of WT cell line DNA background or greater (data not shown).

Table 12 Detection of Plasma Samples spiked with DNA Blends by the KRAS Mutation Test v2 (LSR)

		Copies/mL				
		150	100	75	50	25
Gene-Exon	**Mutation**	**Hit Rate**				
2	G12C	100%	100%	100%	100%	100%
2	G12D	100%	100%	100%	85%	5%
2	G12V	100%	100%	100%	100%	100%
2	G13D	100%	100%	95%	50%	0%
3	A59T	100%	100%	95%	60%	0%
3	Q61Hc	100%	100%	100%	100%	100%
3	Q61L	100%	100%	100%	95%	40%
4	K117Nc	100%	100%	100%	100%	90%
4	A146T	100%	100%	100%	100%	55%

Correlation to reference method

Two milliliters (2 mL) of plasma for 106 clinical plasma samples (61 Colorectal Cancer Plasma Samples and 45 Non-small Cell Lung Cancer Plasma Samples) were extracted and tested. Specimen DNA was characterized by Illumina® MiSeq for mutation and percent mutant variant. The same DNA for each sample was tested using the **KRAS Mutation Test v2 Plasma (LSR)**.

Table 13 LSR and MiSeq Method Correlation Summary

Correlation		MiSeq	
		+	-
KRAS v2	+	18	0
	-	3	85

PPA = 85.7% (18/21)
NPA = 100% (85/85)
Overall Agreement = 97.2% (103/106)

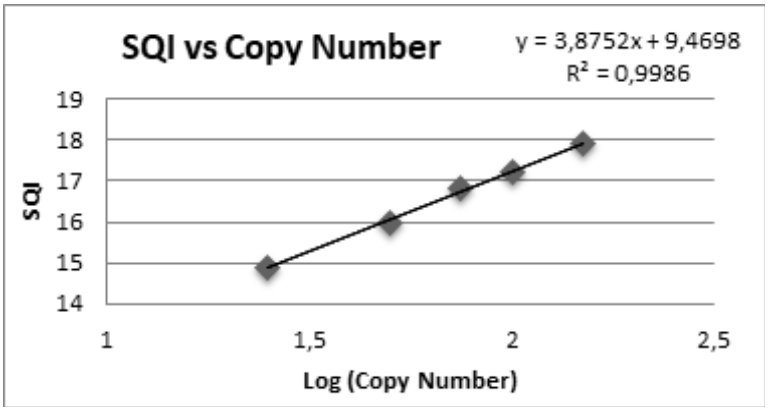
Repeatability

Sheared cell line blends were spiked into 2 mL of normal donor plasma at 2X LOD, as determined by the LOD study, per master mix representing the most frequently occurring mutations in KRAS. Conditions: 3 operators, 2 z480 instruments, testing over 3 days, 2 duplicates per operator. Specimens: G12D mutant specimen (MMx1), G13D mutant specimen (MMx2), and G12C mutant specimen (MMx3). A total of 108 out of 108 genotype calls gave the expected results, thus demonstrating a correct call accuracy of 100%.

Correlation of semi-quantitative index to copy number

SQI values correlate to copy number, as shown in the example below.

Figure 3 Correlation of SQI to Copy Number



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