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

BRAF/NRAS Mutation Test (LSR)

24 reactions

P/N: 07659962001

Version 04

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BRAF/NRAS Mutation Test (LSR): Intended Use

The **BRAF/NRAS Mutation Test (LSR)** is an allele-specific, real-time PCR test for the qualitative detection and identification of exon 11 and 15 mutations in the proto-oncogene B-Raf (BRAF) gene and exon 2, 3, and 4 mutations in the neuroblastoma RAS viral oncogene homolog (NRAS) gene from formalin-fixed, paraffin-embedded tissue (FFPET) or plasma samples. 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 **BRAF/NRAS Mutation Test (LSR)** is based on two major 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 36 unique mutations at a percent mutation of 5% or greater, unless otherwise indicated. The list of mutations is reviewed 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 BRAF/NRAS Mutation Test (LSR) is designed to detect the following mutations

BRAF				
Exon	**Mutation Reported**	**Mutation**	**Nucleic Acid Sequence**	**COSMIC ID**
11	BRAF Exon 11	G466A	c.1397G>C	452
11	BRAF Exon 11	G466V	c.1397G>T	451
11	BRAF Exon 11	G469A	c.1406G>C	460
11	BRAF Exon 11	G469R	c.1405G>A	457
11	BRAF Exon 11	G469V	c.1406G>T	459
15	BRAF V600E/E2/D	V600E	c.1799T>A	476
15	BRAF V600E/E2/D	V600E2	c.1799_1800TG>AA	475
15	BRAF V600E/E2/D	V600D	c.1799_1800TG>AT	477
15	BRAF V600K	V600K	c.1798_1799GT>AA	473
15	BRAF V600R	V600R	c.1798_1799GT>AG	474
15	BRAF K601E	K601E	c.1801A>G	478
NRAS				
Exon	**Mutation Reported**	**Mutation**	**Nucleic Acid Sequence**	**COSMIC ID**
2	G12X	G12A	c.35G>C	565
2	G12X	G12C	c.34G>T	562
2	G12X	G12D	c.35G>A	564
2	G12X	G12R	c.34G>C	561
2	G12X	G12S	c.34G>A	563
2	G12X	G12V	c.35G>T	566
2	G13X	G13A	c.38G>C	575
2	G13X	G13C	c.37G>T	570
2	G13X	G13D	c.38G>A	573
2	G13X	G13R	c.37G>C	569
2	G13X	G13S	c.37G>A	571
2	G13X	G13V	c.38G>T	574
2	A18T	A18T	c.52G>A	577
3	Q61X	Q61Ht	c.183A>T	585
3	Q61X	Q61Hc	c.183A>C	586
3	Q61X	Q61K	c.181C>A	580
3	Q61X	Q61L	c.182A>T	583
3	Q61X	Q61P	c.182A>C	582
3	Q61X	Q61R	c.182A>G	584
3	Other NRAS Ex3/4	A59D	c.176C>A	253327
3	Other NRAS Ex3/4	A59T	c.175G>A	578
3	Other NRAS Ex3/4	K117Nc	c.351G>C	N/A
3	Other NRAS Ex3/4	K117Nt	c.351G>T	N/A
3	Other NRAS Ex3/4	A146T	c.436G>A	27174
3	Other NRAS Ex3/4	A146V	c.437C>T	4170228

Reference Sequences

Please refer to the following sources for reference sequences for both BRAF and NRAS.

BRAF: LRG 299
NRAS: LRG 92

PCR amplification

Target selection

The BRAF/NRAS Mutation Test (LSR) uses primers that define specific base-pair sequences for each of the targeted mutations. Amplification occurs only in the regions of the BRAF or NRAS genes between the primers; the entire gene is not amplified. BRAF sequences range from 101 – 120 base pairs. NRAS sequences range from 94 – 121 base pairs.

Target amplification

A derivative of *Thermus* species Z05-AS1 DNA polymerase is utilized for target amplification. First, the PCR reaction mixture is heated to denature the genomic DNA and expose the primer target sequences. As the mixture cools, the upstream and downstream primers anneal to the target DNA sequences. The Z05 DNA polymerase, in the presence of divalent metal ion and excess dNTP, extends each annealed primer, thus synthesizing a second DNA strand. This completes the first cycle of PCR, yielding a double stranded DNA copy which includes the targeted base-pair regions of the BRAF and NRAS genes. 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 **BRAF/NRAS Mutation Test (LSR)** kit utilizes real-time PCR technology. Each target-specific, oligonucleotide probe in the reaction is labeled with a fluorescent dye that serves as a reporter, and with a quencher molecule that absorbs (quenches) fluorescent emissions from the reporter dye within an intact probe. During each cycle of amplification, probe complementary to the single-stranded DNA sequence in the amplicon binds and is subsequently cleaved by the 5' to 3' nuclease activity of the Z05-AS1 DNA Polymerase. Once the reporter dye is separated from the quencher by this nuclease activity, fluorescence of a characteristic wavelength can be measured when the reporter dye is excited by the appropriate spectrum of light. Three different reporter dyes are used to label the mutations targeted by the test. Amplification of the targeted BRAF and NRAS 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 **BRAF/NRAS Mutation Test (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.

SECTION A: FOR TESTING WITH FFPET SAMPLES

Sample preparation

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

Note: The information below applies only to the cobas® DNA Sample Preparation Kit (P/N: 05985536190)

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. 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 by 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 **BRAF/NRAS Mutation Test (LSR)** kit.

Materials and reagents

Materials and reagents provided

Table 2 Contents of the BRAF/NRAS Mutation Test (LSR) Kit

Kit	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning
BRAF/NRAS Mutation Test (LSR) 24 Tests (P/N: 07659962001)	**MMX-1** BRAF/NRAS Mutation Test (LSR) Master Mix 1 White Cap	2 x 0.48 mL	N/A
	MMX-2 BRAF/NRAS Mutation Test (LSR) Master Mix 2 Brown Cap	2 x 0.48 mL	N/A
	MMX-3 BRAF/NRAS Mutation Test (LSR) Master Mix 3 Blue Cap	2 x 0.48 mL	N/A
	MgAc (Magnesium acetate) Yellow Cap	2 x 0.6 mL	N/A
	BRAF/NRAS MC (BRAF/NRAS 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 Precautions

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. Avoid microbial and DNA contamination of reagents.
5. Dispose of unused reagents and waste in accordance with country, federal, state and local regulations. Do not use kits after their expiration dates.
6. Do not pool reagents from different kits or lots.
7. 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.
8. 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.
9. 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.
10. All disposable items are for one time use. Do not reuse.
11. Do not use disposable items beyond their expiration date.
12. 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.
13. 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.
14. The use of sterile disposable DNase-free pipettor tips is recommended.
15. **Specific to the cobas® DNA Sample Preparation Kit (P/N: 05985536190):**
    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.
16. **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 and Handling Requirements

1. **cobas® DNA Sample Preparation Kit (P/N: 05985536190)**
   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. **BRAF/NRAS Mutation Test (LSR) (P/N: 07659962001)**
   a. Store **MMX-1**, **MMX-2**, **MMX-3**, **MgAc**, **MC**, and **SD** at 2ºC to 8°C.

   Note: ***The BRAF/NRAS Mutation Test (LSR) amplification and detection reagent kit shelf life shall be 12 months from date of manufacturing if unopened and stored at 2-8°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. 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.

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)	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 2 - 1000 µL)	Any vendor
Aerosol barrier or positive displacement DNase-free tips	Any vendor
Pipet-Aid™*	Drummond 4-000-100 or equivalent
Bench top microcentrifuge* (capable of 20,000 x *g*)	Eppendorf 5430 or 5430R or equivalent
Two dry heat blocks capable of heating microcentrifuge tubes to 56ºC and 90ºC*	Any vendor
Safe-Lock™ microcentrifuge tubes (1.5mL, sterile, RNase/ DNase free, PCR grade)	Eppendorf 022363204 or equivalent
Microcentrifuge tube racks	Any vendor
Spectrophotometer for measuring DNA concentration*	Any vendor
Vortex mixer*	Any vendor
Disposable gloves, powder-free	Any vendor
Calibrated thermometers for dry heat block*	Any vendor
Waterbath* capable of maintaining 37ºC	Any vendor
Single edged blade or similar	Any vendor

* All equipment should be maintained according to the manufacturer's instructions.
For more information regarding the materials sold separately, contact your local Roche representative.

Instrumentation and software required but not provided

Required Instrumentation and Software, Not Provided
cobas z 480 Analyzer
cobas® 4800 system User Defined Workflow (UDF)

Suggested handling requirements

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 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

- It is not recommended to 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 intended for one time use. Do not reuse.
- All equipment should be properly maintained according to the manufacturer's instructions.

Disposal

- **DNA TLB**, **DNA EB**, **MgAc**, **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.
- Dispose of unused reagents and waste in accordance with country, federal, state and local regulations.

Spillage and cleaning

- If spills occur on the **cobas**® 4800 instrument, follow the instructions in the 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.

Specific to the cobas® DNA Sample Preparation Kit (P/N: 05985536190)

- **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.
- 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.
- 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.
- **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.

Sample collection, transport, and storage

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

Sample collection

FFPET samples may be used with the **BRAF/NRAS Mutation Test (LSR)**.

Sample transport, storage, and stability

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

Test procedure

Running the test

Table 4 Workflow Steps: BRAF/NRAS Mutation Test (LSR) with cobas® DNA Sample Preparation Kit

1	Start the system
2	Perform instrument maintenance
3	Remove samples and reagents from storage
4	Deparaffinize samples
5	Perform DNA isolation
6	Elute DNA
7	Prepare amplification reagents
8	Load microwell plate with amplification reagents
9	Load microwell plate with sample
10	Seal microwell plate
11	Load microwell plate on the **cobas z** 480 analyzer
12	Enter the run and specimen information in the UDF software and start the run
13	Export raw data file when PCR reaction is complete
14	Upload data file to Data Analysis Portal on Oncology Life Science Research Kits Website
15	Review results

Instructions for use

Note: Only FFPET sections of 5-micron thickness containing at least 10% tumor content by area are to be used in the BRAF/NRAS Mutation Test (LSR). Any sample containing less than 10% tumor content by area should be macrodissected 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 **BRAF/NRAS Mutation Test (LSR)** contains sufficient reagents for 8 runs of 3 samples (plus controls) for a maximum of 24 samples per kit.

Workflow

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

Note: If using a DNA isolation method other than the cobas® DNA Sample Preparation Kit (P/N: 05985536190), 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 Safe-Lock™ 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 of reconstituted **PK** is required for each sample).
Wash Buffer I **(WB I)**	Prepare working **WB I** by adding 15 mL of absolute ethanol to the bottle of **WB I**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB I** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.
Wash Buffer II **(WB II)**	Prepare working **WB II** by adding 50 mL of absolute ethanol to the bottle of **WB II**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB II** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.

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

Deparaffinization of FFPET sections mounted on slides

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

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

1. Add a slide with a mounted 5-micron FFPET section to a container with sufficient xylene to cover the tissue; soak for 5 minutes.
2. Transfer the slide to a container with sufficient absolute ethanol to cover the tissue; soak for 5 minutes.
3. Remove the slide from the ethanol and allow the section to air dry completely (5 to 10 minutes).
4. Perform macro-dissection if the sample contains less than 10% tumor content by area.
5. Label one 1.5 mL Safe-Lock™ microcentrifuge tube for each sample with the sample identification information.
6. Add 180 µL **DNA TLB** to the 1.5-mL Safe-Lock™ microcentrifuge tube.
7. Add 70 µL of reconstituted PK to the Safe-Lock™ microcentrifuge tube containing **DNA TLB**.
8. Scrape the tissue off the slide and into the Safe-Lock™ microcentrifuge tube. Immerse the tissue in the **DNA TLB/PK** mixture.
9. Continue with Step 1 of the **DNA Isolation procedure**.

Deparaffinization of FFPET sections not mounted on slides

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

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

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

DNA isolation procedure

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

1. Vortex the tubes containing the sample/**DNA TLB/PK** mixture and the Negative Control (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 Safe-Lock™ microcentrifuge tube).

Note: During the incubation, label the required number of elution tubes (1.5 mL Safe-Lock™ 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 (Safe-Lock™1.5 mL microcentrifuge tube) pre-labeled with sample or control identification. Discard the flow-through from the used **CT** into chemical waste, and properly dispose of the used **CT**.
20. Add 100 µL **DNA EB** to the center of each **FT** membrane without touching the **FT** membrane.
21. Incubate the **FT** with elution tube at 15°C to 30°C for 5 minutes.
22. Centrifuge the **FT** with elution tube at 8,000 x *g* for 1 minute to collect eluate into the elution tube. Properly dispose of the used **FT**.
23. Close the cap on the elution tube. The elution tube contains the DNA Stock. Proceed to Step 1 in the **DNA Quantitation** section.

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

DNA Quantitation

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

Note: The DNA Stock from the processed negative control (NC) does not need to be measured.

3. The DNA Stock concentration from the samples must be > 2 ng/µL to perform the **BRAF/NRAS Mutation Test (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 BRAF/NRAS Mutation Test (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.

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 BRAF/NRAS Mutation Test (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 spread across three consecutive columns in one row in order to generate a response.

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

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

Dilution calculation of sample DNA stock

Dilution calculation for DNA stock concentrations from 2 ng/µL to 36 ng/µL

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

Note: Three amplification/detections are run for each sample requiring a total volume of 75 µL (25 µL for each of three reactions) of a 2 ng/µL dilution of DNA Stock (total of 150 ng DNA).

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

   µL of DNA stock = (90 µL x 2 ng/µL) ÷ DNA Stock concentration [ng/µL]

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

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 Safe-Lock™ 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	**6.5 µL**	26	32.5	39	58.5	78	97.5	117	136.5	156	175.5
Total Vol. for Each Working MMX (µL)		**106**	**132.5**	**159**	**238.5**	**318**	**397.5**	**477**	**556.5**	**636**	**715.5**

* 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.
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 BRAF/NRAS FFPET (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 **BRAF/NRAS FFPET**.
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., BRAF/NRAS FFPET and KRASv2 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 Ct 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.***

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 **BRAF/NRAS FFPET** 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 BRAF/NRAS Mutation Test (LSR)

Test Result	Mutation Result	Interpretation
Mutation Detected	BRAF V600E/E2/D (BRAF V600E, V600E2, V600D) BRAF V600K BRAF V600R** BRAF K601E BRAF Exon11 (G466A, G466V, G469A, G469R, G469V) NRAS G12X (G12A, G12C, G12D, G12R, G12S, G12V) NRAS G13X (G13A, G13C, G13D, G13R, G13S, G13V) NRAS A18T NRAS Q61X (Q61Ht, Q61Hc, Q61K, Q61L, Q61P, Q61R) NRAS Other Ex 3 and 4 (A59D, A59T, K117Nc, K117Nt, A146T, A146V)	Mutation detected in specified targeted region.
No Mutation Detected (NMD)*	**N/A**	Mutation not detected in targeted 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 BRAF or NRAS regions because results depend on percent mutant sequences, adequate sample integrity, absence of inhibitors, and sufficient DNA to be detected.
** Cross-reactivity to BRAF V600M has been observed in analytical performance studies. Please see **Procedural limitations** section.

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, R712, 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, R713, 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, R714, 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, R715, 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, R776, 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, R777, 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, R806, 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, R809, R810, 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 that an atypically low Internal Control Ct value was observed for the sample. This error may occur if the PCR mixture is overloaded with concentrated genomic DNA.
R797, R802, R807, R817, R822, R827, R837, R842, R847	No target could be detected.	Repeat the run. These flag codes indicate that a negative result occurred for the sample (i.e. sample may have not been added to one or more wells).
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 Samples. 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 samples with invalid results

1. Repeat dilution of the invalid sample DNA stock starting from "Dilution Calculation of Sample DNA Stock" and "Sample Dilution" procedures in the **Amplification and detection** section.
2. After performing the DNA stock dilution to 2 ng/µL as described in "Sample Dilution", continue with "Preparation of working master mix (MMX-1, MMX-2 and MMX-3)" and the remainder of the amplification and detection procedure.

Note: If the sample remains invalid after retesting or there was not enough DNA stock to prepare another dilution in Retesting of Samples with Invalid Results, repeat the entire test procedure for that sample, starting with deparaffinization and DNA Isolation using one new 5-micron FFPET slide.

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 **BRAF/NRAS Mutation Test (LSR)** was tested with melanoma, lung, and colorectal cancer FFPET samples.
3. The **BRAF/NRAS Mutation Test (LSR)** was verified using the **cobas**® DNA Sample Preparation Kit (P/N: 05985536190).
4. BRAF V600M may be detected by the BRAF V600R reaction due to cross-reactivity. BRAF V600M plasmid DNA was detected at 100 copies per reaction.
5. 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.
6. Reliable results are dependent on adequate sample fixation, transport, storage and processing.
7. Follow the procedures in this Package Insert to ensure satisfactory results.
8. The effects of other potential variables such as sample fixation variables have not been evaluated.
9. The addition of AmpErase enzyme into the **BRAF/NRAS Mutation Test (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.
10. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas**® 4800 system.
11. Only the **cobas z** 480 analyzer was verified for use with this product. No other thermal cycler with real-time optical detection can be used with this product at this time.
12. Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to the next-users perform method correlation studies in their laboratory to qualify technology differences.
13. The presence of PCR inhibitors may cause false negative or invalid results.
14. The **BRAF/NRAS Mutation Test (LSR)** was verified for use with 50 ng of DNA per reaction well. DNA input amounts other than 50 ng per reaction well are not recommended.

Analytical Performance Evaluation

The following data demonstrate the analytical performance of the **BRAF/NRAS Mutation Test (LSR)**.

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

Limit of Detection Using Plasmid DNA Blends

DNA isolated from melanoma FFPET samples with NRAS or BRAF mutations were blended with DNA isolated from NRAS and BRAF wildtype melanoma FFPET samples to achieve blends targeting 20%, 10%, 5%, and 2.5% mutation levels in 50ng DNA as determined by Illumina® MiSeq sequencing. Two duplicates for each mutation level were tested using one pilot lot kit for each sample. 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.

Table 9 Limit of Detection for the BRAF/NRAS Mutation Test (LSR) using FFPET DNA Blends

Gene-Exon	Mutation	Nucleic Acid Sequence	Cosmic ID	Samples detected/tested per mutation level			
				20%	10%	5%	2.5%
BRAF-Exon 15	V600E	c.1799T>A	476	9/9	12/12	13/13	14/15
BRAF-Exon 15	V600K	c.1798_1799GT>AA	356	9/9	10/10	10/10	11/11
BRAF-Exon 15	V600R	c.1798_1799GT>AG	474	2/2	2/2	2/2	2/2
BRAF-Exon 15	K601E	c.1801A>G	478	1/1	1/1	1/1	0/1
NRAS-Exon 3	Q61K	c.181C>A	580	6/6	6/6	7/7	7/7
NRAS-Exon 3	Q61R	c.182A>G	584	2/2	3/3	3/3	4/4
NRAS-Exon 2	G13D	c.38G>A	573	1/1	1/1	1/1	0/1
NRAS-Exon 2	G13V	c.38G>T	574	2/2	2/2	2/2	2/2
Total number of samples				**32/32**	**37/37**	**39/39**	**40/43**

- 100% detection rate for samples with 20%, 10%, and 5% mutant DNA in a background of WT genomic DNA.
- 93% detection rate (40/43 samples) at 2.5% mutant DNA in a background of WT genomic DNA.

Limit of Detection Using Plasmid DNA Blends

Plasmid DNA constructs were blended with wildtype DNA to prepare percent mutant DNA samples ranging from 1% to 10%. A total of 10 replicates for each plasmid blend was tested with a DNA input of 50ng using a pilot lot kit.

Table 10 Plasmid DNA Sample DNA Blends

Gene-Exon	Mutation	Nucleic Acid Sequence	Cosmic ID
BRAF-Exon 15	V600E	c.1799T>A	476
BRAF-Exon 15	V600E2	c.1799_1800TG>AA	475
BRAF-Exon 15	V600D	c.1799_1800TG>AT	477
BRAF-Exon 15	V600K	c.1798_1799GT>AA	356
BRAF-Exon 15	V600R	c.1798_1799GT>AG	474
BRAF-Exon 15	K601E	c.1801A>G	478
BRAF-Exon 11	G466A	c.1397G>C	452
BRAF-Exon 11	G466V	c.1397G>T	451
BRAF-Exon 11	G469A	c.1406G>C	460
BRAF-Exon 11	G469R2	c.1405G>A	457
BRAF-Exon 11	G469V	c.1406G>T	459
NRAS-Exon 2	G12A	c.35G>C	565
NRAS-Exon 2	G12C	c.34G>T	562
NRAS-Exon 2	G12D	c.35G>A	564
NRAS-Exon 2	G12R	c.34G>C	561
NRAS-Exon 2	G12S	c.34G>A	563
NRAS-Exon 2	G12V	c.35G>T	566
NRAS-Exon 2	G13A	c.38G>C	575
NRAS-Exon 2	G13C	c.37G>T	570
NRAS-Exon 2	G13D	c.38G>A	573
NRAS-Exon 2	G13R	c.37G>C	569
NRAS-Exon 2	G13S	c.37G>A	571
NRAS-Exon 2	G13V	c.38G>T	574
NRAS-Exon 2	A18T	c.52G>A	577
NRAS-Exon 3	Q61Ht	c.182A>T	585
NRAS-Exon 3	Q61Hc	c.183A>C	586
NRAS-Exon 3	Q61K	c.181C>A	580
NRAS-Exon 3	Q61L	c.182A>T	583
NRAS-Exon 3	Q61P	c.182A>C	582
NRAS-Exon 3	Q61R	c.182A>G	584
NRAS-Exon 3	A59D	c.176C>A	253327
NRAS-Exon 3	A59T	c.175G>A	578
NRAS-Exon 4	K117Nc	c.?	N/A
NRAS-Exon 4	K117Nt	c.?	N/A
NRAS-Exon 4	A146T	c.436G>A	27174
NRAS-Exon 4	A146V	c437C>T	4170228

All mutations tested had an analytical sensitivity of at least 5% mutant.

Correlation to Reference Method

Comparison testing of 164 FFPET samples (94 melanoma, 48 CRC, and 22 NSCLC) using one pilot lot kit and MiSeq sequencing was performed to determine the overall agreement between methods.

Table 11 BRAF/NRAS Mutation Test (LSR) vs. MiSeq Sequencing

		MD	NMD	Total
BRAF/NRAS Mutation Test	MD	45	0	45
	NMD	0	116	116
	Invalid	3	0	3
	Total	48	116	164

Overall agreement = 98.2%
MD = Mutation Detected
NMD = No Mutation Detected

The comparison between the **BRAF/NRAS Mutation Test** and Illumina® MiSeq sequencing evaluated nine sample mutations: BRAF V600E, BRAF V600K, BRAF V600R, BRAF K601E, BRAF G469A, NRAS Q61R, NRAS Q61K, NRAS G13D, and NRAS G13V.

Table 12 Sample Mutations Evaluated in the Method Correlation Study

Mutation	Total
BRAF V600E	16
BRAF V600R	2
BRAF V600K	10
BRAF K601E	1
BRAF G469A	1
NRAS G13D	1
NRAS G13V	2
NRAS Q61K	7
NRAS Q61R	5
Number of BRAF mutants	30
Number of NRAS mutants	15
Total number of mutant samples	43
Total number of wildtype	48
Total number of samples tested	91

Three additional samples were invalid by **BRAF/NRAS Mutation Test (LSR)**. Repeat testing by MiSeq sequencing resulted in discrepant results using this method.

Repeatability

Repeatability of the **BRAF/NRAS Mutation Test** was assessed using six FFPET samples including one wildtype sample and five mutant samples. Three BRAF mutant samples had the following mutations - V600E, V600K, and K601E. Two NRAS mutant samples had the following mutations - Q61R and G13D. Each sample was tested in duplicate with one pilot lot kit, by 2 operators, and 2 instruments over four days. A total of 96 out of 96 replicates (48 replicates per operator) gave the expected results, thus demonstrating a correct call accuracy of 100%.

SECTION B: FOR TESTING WITH PLASMA SAMPLES

Sample preparation

Note: The cobas® cfDNA Sample Preparation Kit (P/N: 07247737190) is strongly preferred for use with the BRAF/NRAS Mutation Test (LSR) kit, though it is not required. Alternative sample preparation methods should be validated for use with the BRAF/NRAS Mutation Test (LSR).

Note: The information below applies only to the cobas® cfDNA Sample Preparation Kit (P/N: 07247737190)

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 **BRAF/NRAS Mutation Test (LSR)**.

Materials and reagents

Materials and reagents provided

Table 13 Contents of the BRAF/NRAS Mutation Test (LSR) Kit

Kit	Components and Reagent Ingredients	Quantity per Test	Safety Symbol and Warning
BRAF/NRAS Mutation Test (LSR) 24 Tests (P/N: 07659962001)	**MMX-1** (BRAF/NRAS Master Mix 1) White Cap	2 x 0.48 mL	N/A
	MMX-2 (BRAF/NRAS Master Mix 2) Brown Cap	2 x 0.48 mL	N/A
	MMX-3 (BRAF/NRAS Master Mix 3) Blue Cap	2 x 0.48 mL	N/A
	MgAc (Magnesium acetate) Yellow Cap	2 x 0.6 mL	N/A
	BRAF/NRAS MC (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 Precautions

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. Avoid microbial and DNA contamination of reagents.
5. Dispose of unused reagents and waste in accordance with country, federal, state and local regulations. Do not use kits after their expiration dates.
6. Do not pool reagents from different kits or lots.
7. Gloves must be worn and must be changed between handling samples and reagents to prevent contamination.
8. 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.
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.
10. 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.
11. All disposable items are for one time use. Do not reuse.
12. Do not use disposable items beyond their expiration date.
13. 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.
14. 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.
15. The use of sterile disposable DNase-free pipettor tips is recommended.
16. **Specific to 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** contain a non-ionic detergent which is an irritant to mucous membranes. Avoid contact with eyes, skin, and mucous membranes.
17. **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 and Handling Requirements

1. **cobas® cfDNA Sample Preparation Kit (P/N: 07247737190)**

    a. With the exception of the **PK** reagent, do not freeze reagents.

Store **DNA PBB**, **WB I**, **WB II**, **DNA EB**, **PK**, **HPEA FT** unit (High Pure Extension Assembly Unit) and **CT** at 15ºC to 30ºC. Once opened, **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.

b. After addition of sterile, nuclease free water to **PK**, store unused reconstituted **PK** in 1.1mL aliquots at -20ºC. Once reconstituted, **PK** must be used within 90 days or until the expiration date, whichever comes first.

c. 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. **BRAF/NRAS Mutation Test (LSR) (P/N: 07659962001)**

    a. Store **MMX-1**, **MMX-2**, **MMX-3**, **MgAc**, **MC**, and **SD** at 2ºC to 8°C.

    Note: The BRAF/NRAS Mutation Test (LSR) amplification and detection reagent kit shelf life shall be 12 months from date of manufacturing if unopened and stored at 2-8°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. 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.

Additional materials required

Table 14 Materials Needed but Not Provided

Materials	P/N
Portable media drive (*e.g.*, flash drive)	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

Materials	P/N
Table top centrifuge* (capable of 6,000 x *g* while holding 50mL conical tubes in a swing-bucket rotor)	Eppendorf model 5810 or equivalent
15-mL Sterile conical plastic tubes	Any vendor
Safe-Lock™ microcentrifuge tubes (1.5mL, sterile, RNase/ DNase free, PCR grade)	Eppendorf 022363204 or equivalent
Conical and microcentrifuge tube racks	Any vendor
Vortex mixer*	Any vendor
Disposable gloves, powder-free	Any vendor

* All equipment should be maintained according to the manufacturer's instructions.
For more information regarding the materials sold separately, contact your local Roche representative.

Instrumentation and software required but not provided

Required Instrumentation and Software, Not Provided
cobas z 480 Analyzer
cobas® 4800 system User Defined Workflow (UDF)

Suggested handling requirements

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 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

- It is not recommended to 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 intended for one time use. Do not reuse.
- All equipment should be properly maintained according to the manufacturer's instructions.

Disposal

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

Spillage and cleaning

- If spills occur on the **cobas**® 4800 instrument, follow the instructions in the 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.

Specific to the cobas® cfDNA Sample Preparation Kit (P/N: 07247737190)

- **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.
- 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.
- 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.
- **DNA PBB** contain a non-ionic detergent which is an irritant to mucous membranes. Avoid contact with eyes, skin, and mucous membranes.

Sample collection, transport, and storage

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

Sample collection

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.

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

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

Table 14 Workflow Steps: BRAF/NRAS Mutation Test (LSR) 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	Prepare amplification reagents
8	Load microwell plate with amplification reagents
9	Load microwell plate with sample
10	Seal microwell plate
11	Load microwell plate on the **cobas z** 480 analyzer
12	Enter the run and specimen information in the UDF software and start the run
13	Export raw data file when PCR reaction is complete
14	Upload data file to Data Analysis Portal on Oncology Life Science Research Kits Website
15	Review results

Instructions for use

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 **BRAF/NRAS Mutation Test (LSR)** contains sufficient reagents for 8 runs of 3 samples (plus controls) for a maximum of 24 samples per kit.

Workflow

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

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

Table 15 Reagent Preparation (if using the cobas® cfDNA 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 Safe-Lock™ 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 (250 µL of reconstituted **PK** is required for each sample).
Wash Buffer I **(WB I)**	Prepare working **WB I** by adding 15 mL of absolute ethanol to the bottle of **WB I**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB I** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.
Wash Buffer II **(WB II)**	Prepare working **WB II** by adding 50 mL of absolute ethanol to the bottle of **WB II**. Mix by inverting the bottle 5 to 10 times. Note on the bottle that ethanol has been added and the date. Store working **WB II** at 15°C to 30°C for up to 90 days or until the expiration date, whichever comes first.

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

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.
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.

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 2**.

Figure 2 Plate layout for the BRAF/NRAS Mutation Test (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 spread across three consecutive columns in one row in order to generate a response.

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

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

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 MMXs, one containing **MMX-1**, one containing **MMX-2**, and the other containing **MMX-3** in separate 1.5 mL Safe-Lock™ 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 16** 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 16 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	**6.5 µL**	26	32.5	39	58.5	78	97.5	117	136.5	156	175.5
Total Vol. for Each Working MMX (µL)		**106**	**132.5**	**159**	**238.5**	**318**	**397.5**	**477**	**556.5**	**636**	**715.5**

* 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

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 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 MC in wells A01, A02 and A03, and 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 2 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 BRAF/NRAS 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 perform 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 **BRAF/NRAS 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 MC in positions A01 – A03 and a NC in positions B01 – B03. However, if users choose to run more than one test parameter on the plate (e.g., BRAF/NRAS Plasma and KRASv2 FFPET), a new row of test-specific Mutant Controls and Negative Controls must be included at the top of a new column, with the MC in the first row and the NC in the second.***

Data Analysis

Note: ***It is NOT recommended to use Ct 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.***

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 **BRAF/NRAS 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 17**.

Table 17 Result interpretation for the BRAF/NRAS Mutation Test (LSR)

Test Result	Mutation Result	Interpretation
Mutation Detected	BRAF V600E/E2/D (BRAF V600E, V600E2, V600D) (SQI) BRAF V600K (SQI) BRAF V600R** (SQI) BRAF K601E (SQI) BRAF Exon11 (G466A, G466V, G469A, G469R, G469V) (SQI) NRAS G12X (G12A, G12C, G12D, G12R, G12S, G12V) (SQI) NRAS G13X (G13A, G13C, G13D, G13R, G13S, G13V) (SQI) NRAS A18T (SQI) NRAS Q61X (Q61Ht, Q61Hc, Q61K, Q61L, Q61P, Q61R) (SQI) NRAS Other Ex 3 and 4 (A59D, A59T, K117Nc, K117Nt, A146T, A146V) (SQI)	Mutation detected in specified targeted region.
No Mutation Detected (NMD)*	**N/A**	Mutation not detected in targeted 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 BRAF or NRAS regions because results depend on percent mutant sequences, adequate sample integrity, absence of inhibitors, and sufficient DNA to be detected.
** Cross-reactivity to BRAF V600M has been observed in analytical performance studies. Please see **Procedural limitations** section.

Semi Quantitative Index (SQI)

Note: The SQI will be heavily influenced by the pre-analytical methods employed using the BRAF/NRAS Mutation Test (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 18 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, R712, 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, R713, 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, R714, 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, R715, 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, R776, 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, R777, 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).
R797, R802, R807, R817, R822, R827, R837, R842, R847	No target could be detected.	Repeat the run. These flag codes indicate that a negative result occurred for the sample (i.e. sample may have not been added to one or more wells).

Flag Code	Description	Recommended Action
R796, R801, R806, 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, R809, R810, 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.
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 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.
R814, R834, R854	Internal Control out of range.	Repeat the sample. These flag codes indicate that an atypically low Internal Control Ct value was observed for the sample. This error may occur if the PCR mixture is 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 Samples. 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 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 **BRAF/NRAS Mutation Test (LSR)** was tested with melanoma, lung, and colorectal cancer plasma samples.
3. The **BRAF/NRAS Mutation Test (LSR)** was verified using the **cobas**® cfDNA Sample Preparation Kit (Roche P/N: 07247737190)
4. BRAF V600M may be detected by the BRAF V600R reaction due to cross-reactivity. BRAF V600M plasmid DNA was detected at 100 copies per reaction.
5. 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.
6. Reliable results are dependent on adequate sample collection, transport, storage and processing.
7. Follow the procedures in this Package Insert to ensure satisfactory results.
8. The effects of other potential variables such as sample variables have not been evaluated.
9. The addition of AmpErase enzyme into the **BRAF/NRAS Mutation Test (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.
10. Use of this product must be limited to personnel trained in the techniques of PCR and the use of the **cobas**® 4800 system.
11. Only the **cobas z** 480 analyzer was verified for use with this product. No other thermal cycler with real-time optical detection can be used with this product at this time.
12. Due to inherent differences between technologies, it is recommended that, prior to switching from one technology to the next, users perform method correlation studies in their laboratory to qualify technology differences.
13. The presence of PCR inhibitors may cause false negative or invalid results.
14. The **BRAF/NRAS Mutation Test (LSR)** was verified for use with 25µL of DNA stock per reaction well. DNA input volumes lower 25µL per reaction well are not recommended.

Analytical Performance Evaluation

The following data demonstrate the analytical performance of the **BRAF/NRAS Mutation Test (LSR)** in plasma. The data are not intended to demonstrate any clinical performance claims for the test. The **BRAF/NRAS Mutation Test (LSR)** is not intended for diagnostic procedures.

Limit of Detection Using Plasmid DNA Blends

Mutant plasmid DNA samples representing the 36 BRAF and NRAS mutations were added to healthy donor plasma containing sheared wildtype genomic DNA and processed using the **cobas**® cfDNA kit. 100 to 500 mutant plasmid DNA copies for all 36 BRAF and NRAS variants were added to 2mL plasma. Eluates were then blended with sheared wildtype DNA eluates at concentrations ranging from 3200 to 160,000 copies per reaction. Summaries of the observed limits of detection are noted in Tables 19 to 24.

Table 19 BRAF mutations for which 100 DNA copies per mL plasma were detected in the highest sheared WT gDNA concentration

BRAF mutations	WT gDNA concentration (copies/PCR)			
	160000	64000	16000	6400
	mutant copies detected in varying WT DNA			
B V600E	100	100	100	100
B V600K	100	100	100	100
B V600R	100	100	100	100
B G466A	100	100	100	100
B G469A	100	100	100	100
B G469R2	100	100	100	100
B G469V	100	100	100	100

Table 20 BRAF mutations for which 200 DNA copies per mL plasma were detected in the highest sheared WT gDNA concentration

BRAF mutations	WT gDNA concentration (copies/PCR)			
	160000	64000	16000	6400
	mutant copies detected in varying WT DNA			
B V600E2	200	200	200	200
B V600D	200	200	200	200
B K601E	200	200	200	200
B G466V	200	100	100	100

Table 21 NRAS mutations for which 100 DNA copies per mL plasma were detected in the highest sheared WT gDNA concentration

NRAS mutations	WT gDNA concentration (copies/PCR)			
	160000	64000	16000	6400
	mutant copies detected in varying WT DNA			
N G12A	100	100	100	100
N G12C	100	100	100	100
N G12D	100	100	100	100
N G12R	100	100	100	100
N G13A	100	100	100	100
N Q61Ha	100	100	100	100
N Q61K	100	100	100	100
N Q61L	100	100	100	100
N Q61P	100	100	100	100

Table 22 NRAS mutations for which 200 DNA copies per mL plasma were detected in the highest sheared WT gDNA concentration

NRAS mutations	WT gDNA concentration (copies/PCR)			
	160000	64000	16000	6400
	mutant copies detected in varying WT DNA			
N G12S	200	200	200	200
N G12V	200	200	200	200
N G13C	200	200	100	100
N G13D	200	100	100	100
N G13R	200	100	100	100
N G13S	200	100	100	100
N G13V	200	100	100	100
N Q61Hb	200	200	200	100
N Q61R	200	200	100	100
N A18T	200	200	200	200
K117Nc	200	200	200	200

Table 23 Additional NRAS mutations for which 200 DNA copies per mL plasma were detected in the highest sheared WT gDNA concentration

NRAS mutations	WT gDNA concentration (copies/PCR)		
	16000	6400	3200
	mutant copies detected in varying WT DNA		
N A59D	200	200	200
N A59T	200	200	200
N K117Nt	200	200	200
N A146V	200	200	200

Table 24 NRAS A146T mutation for which 250 DNA copies per mL plasma were detected in the highest sheared WT gDNA concentration

NRAS mutations	WT gDNA concentration (copies/PCR)		
	16000	6400	3200
	mutant copies detected in varying WT DNA		
N A146T	250	250	250

Correlation to Reference Method

186 plasma samples from 128 melanoma, 57 CRC, and one normal plasma donor were tested by LSR and the Illumina 2-gene MiSeq assay. Results are noted in **Tables 25** and **26**. cfDNA was extracted using the **cobas**® cfDNA Sample Preparation Kit.

The same eluate from each sample was used for LSR and MiSeq testing.

Table 25 Summary of LSR Results

LSR Result	N	%
NMD	137	73.7%
MUTANT	49	26.3%
BRAF Mutant	39	21.0%
V600E/E2/D	34	18.3%
V600K	4	2.2%
V600R	1	0.5%
NRAS Mutant	8	4.3%
Q61X	4	2.2%
G12X	2	1.1%
G13X	2	1.1%
BRAF+NRAS Mutant	2	1.1%
V600E/E2/D + N Q61X	2	1.1%
TOTAL	**186**	

Table 26 LSR and MiSeq Method Correlation Summary

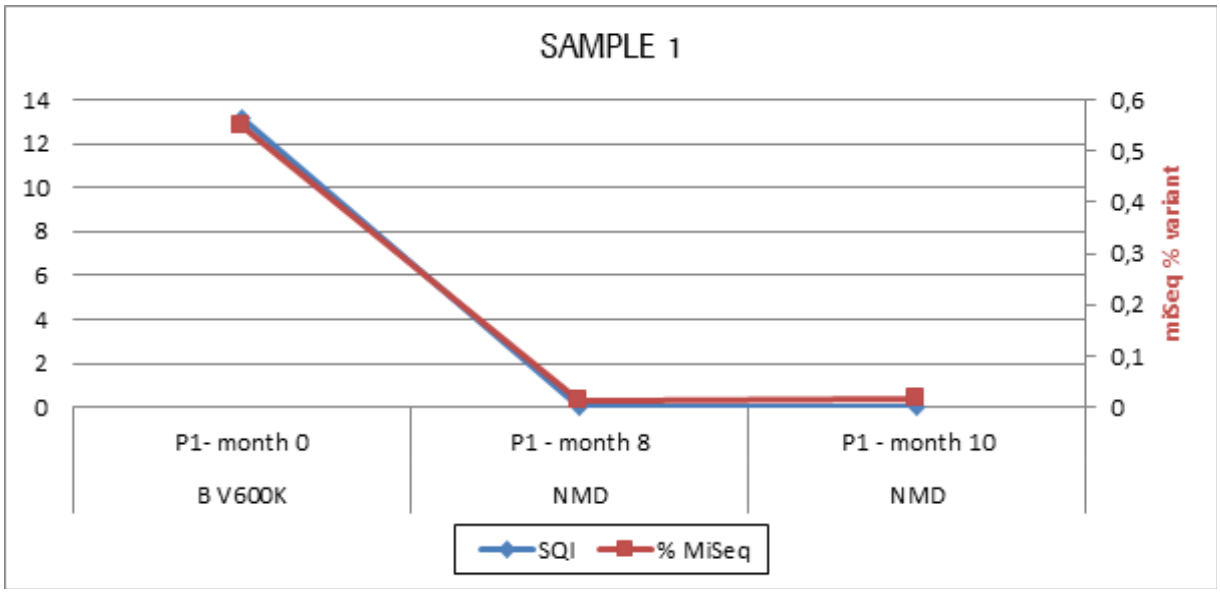
Sample-Wise Agreement		MiSeq	
		+	-
LSR	+	48	0
	-	2	136

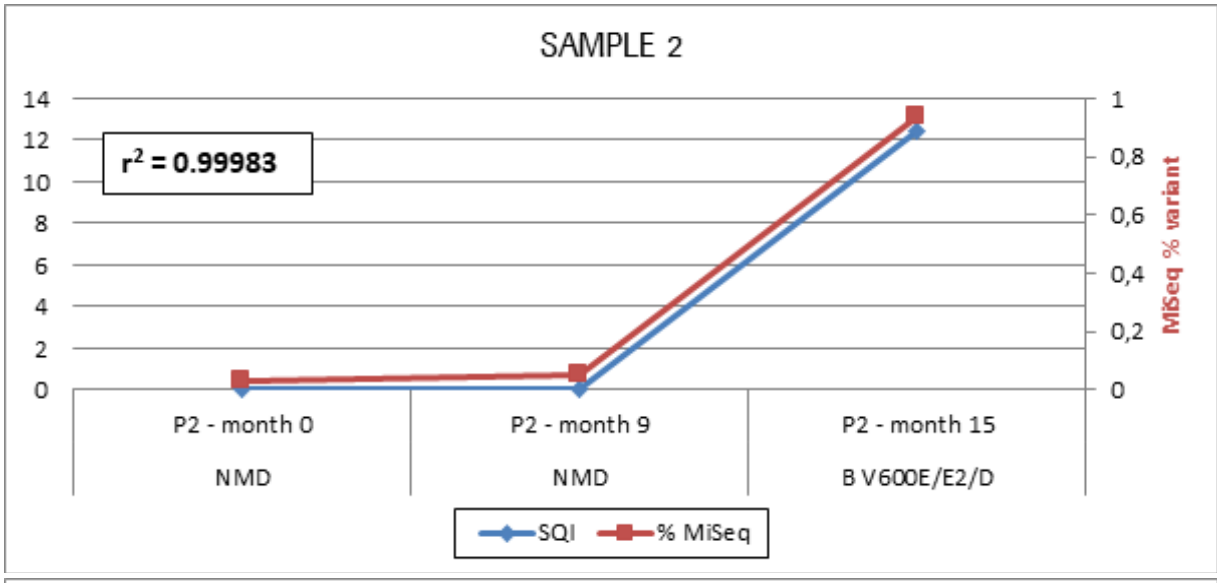
Overall agreement: 184/186 (98.9%)
Negative Agreement: 136/136 (100%)
Positive Agreement: 48/50 (96.0%)

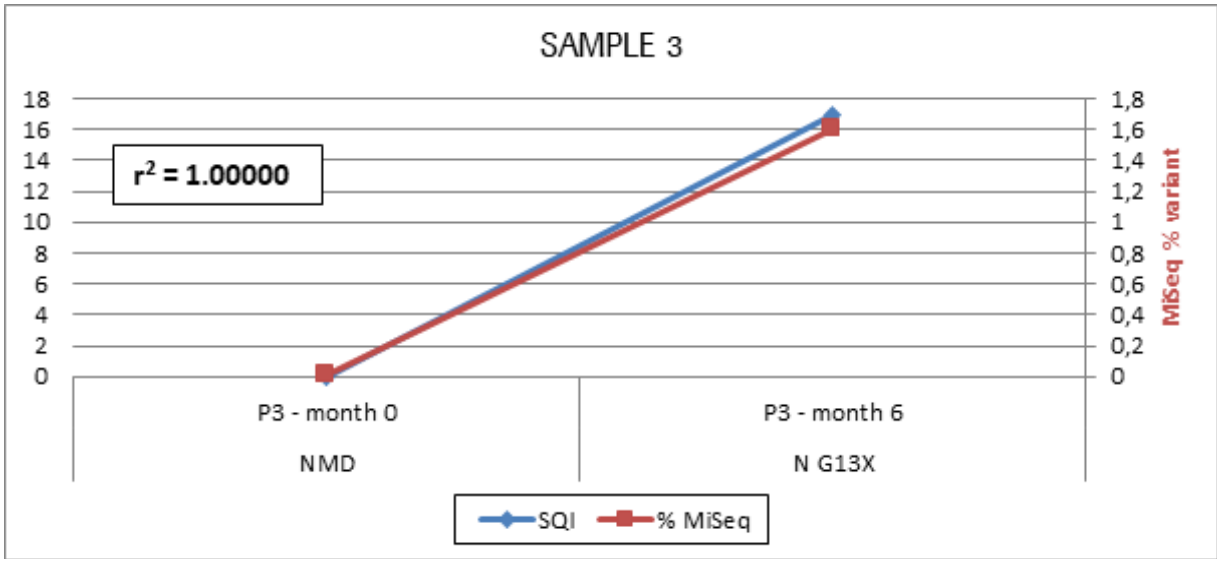
Correlation of Semi-Quantitative Index to % Mutation

Various human, plasma samples were collected across multiple time points and tested with both the **BRAF/NRAS Mutation Test (LSR)** and the in-house MiSeq reference method to assess the concordance between the semi-quantitative index (SQI) and percent mutation. The results of those assessments are listed below (**Figure 3A/B/C**).

Figure 3A/B/C Correlation of SQI to Percent Mutation in Human Plasma Samples.







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