March 2024

dPCR PanCancer Kits Handbook

For parallel detection of multiple somatic mutations with digital PCR

Sample to Insight

Contents

Kit Contents

The dPCR PanCancer kits are shipped in 2 separate boxes. Box 1 contains 20x oligo mixes of each, the PanCancer assay and the reference assay for AP3B1. Box 2 contains the QIAcuity® MasterMix and RNAse-free water to setup the dPCR reaction.

dPCR PanCancer Assay Kit	Box 1 (Assays)	Box 2 (MasterMix)
Cat. nos.	250284, 250287	250284, 250287
Number of Reactions	200*	200*
20x Oligo Mix PanCancer assay	1 vial	
20x Oligo Mix reference assay AP3B1	1 vial	
4x QIAcuity MasterMix		2x 1 mL
RNAse-free water		3x 1.9 mL

* The number of reactions is calculated on the basis of the 40 µL reaction in the 26K dPCR nanoplate.

Shipping and Storage

The dPCR PanCancer kits are shipped in 2 separate boxes. Both boxes are shipped on dry ice.

Both boxes should be stored at –30°C to –15°C in a constant temperature freezer. Under these conditions, the components are stable until expiry date printed on the labels without showing any reduction in performance and quality, unless otherwise indicated on the labels.

The dPCR PanCancer assays in Box 1 are shipped on dry ice and should upon receipt be stored protected from light at –30°C to –15°C in a constant-temperature freezer for long term storage. After reconstitution, the assays are stable for at least 12 months. It is recommended to store the dPCR PanCancer assays in aliquots at –30°C to –15°C to avoid repeated freeze-thaw cycles.

Intended Use

The dPCR PanCancer Kits are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at **www.qiagen.com/safety**, where you can find, view, and print the SDS for each QIAGEN kit and kit component.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of dPCR PanCancer Kits is tested against predetermined specifications to ensure consistent product quality.

Introduction

Somatic mutations are genetic alterations that occur in the cells of the body, excluding reproductive cells, and are not passed on to offspring. The shift toward precision medicine in oncology highlights the importance of identifying specific somatic mutations that drive cancer. Key mutations in genes like serine/threonine-protein kinase B-Raf (BRAF) and the epidermal growth factor receptor (EGFR) play a significant role in guiding therapeutic decisions. Digital PCR has emerged as a powerful technology for precise and quantitative mutation analysis, supporting clinical research.

The dPCR PanCancer Assays are designed to simultaneously detect multiple hallmark mutations in BRAF and EGFR. Each assay targets a range of mutations associated with these genes, facilitating a comprehensive analysis. This simultaneous assessment of BRAF and EGFR mutations streamlines for example pre-screening processes or long-term monitoring, reducing both time and costs. Respective mutations detected by the PanCancer assays are listed in Table 1. Each PanCancer assay contains a reference assay that quantifies the human single copy gene AP3B1 that serves as a reference gene to determine the number of genome copies in the sample.

Table 1. List of mutation targeted by the PanCancer Kits

PanCancer Kit	Mutation aa	Mutation nucleotide	COSMIC ID*
BRAF PanCancer Kit	p.V600K	c.1798_1799delinsAA	COSV56057713
	p.V600R	c.1798_1799delinsAG	COSV56058419
	p.V600E	c.1799_1800delinsAA	COSV56059110
	p.V600E	c.1799T>A	COSV56056643
	p.V600D	c.1799_1800delinsAT	COSV56059623
	p.V600G	c.1799T>G	COSV56080151
	p.V600M	c.1798G>A	COSV56075762
	p.V600R	c.1798_1799delinsCG	COSV56288520

PanCancer Kit	Mutation aa	Mutation nucleotide	COSMIC ID*
EGFR PanCancer Kit	p.K745_E749del	c.2233_2247del	COSV51769442
	p.E746_A750delinsIP	c.2235_2248delinsAATTC	COSV51817953
	p.E746_A750del	c.2235_2249del	COSV51765119
	p.E746_T751delinsIP	c.2235_2251delinsAATTC	COSV51782151
	p.E746_T751delinsI	c.2235_2252delinsAAT	COSV51850034
	p.E746_A750del	c.2236_2250del	COSV51765066
	p.E746_T751delinsA	c.2237_2251del	COSV51769364
	p.E746_T751delinsV	c.2237_2252delinsT	COSV51775936
	p.E746_T751delinsVA	c.2237_2253delinsTTGCT	COSV51771891
	p.E746_S752delinsV	c.2237_2255delinsT	COSV51765862
	p.L747_A750delinsP	c.2238_2248delinsGC	COSV51782279
	p.L747_T751delinsQ	c.2238_2252delinsGCA	COSV51863059
	p.E746_S752delinsD	c.2238_2255del	COSV51772418
	p.L747_E749del	c.2239_2247del	COSV51780076
	p.L747_A750delinsP	c.2239_2248delinsC	COSV51765099
	p.L747_T751delinsP	c.2239_2251delinsC	COSV51765856
	p.L747_S752del	c.2239_2256del	COSV51767308
	p.L747_S752delinsQ	c.2239_2256delinsCAA	COSV51778874
	p.L747_P753delinsQ	c.2239_2258delinsCA	COSV51785746
	p.L747_T751delinsS	c.2240_2251del	COSV51768180
	p.L747_T751del	c.2240_2254del	COSV51766247
	p.L747_A750delinsS	c.2240_2248del	COSV51810296
	p.L747_P753delinsS	c.2240_2257del	COSV51767961

* COSMIC IDs taken from the Catalog of somatic mutations in cancer (COSMIC) database in 11/2023 (**www.cancer.sanger.ac.uk/cosmic**).

Utilizing a carefully optimized digital PCR setup, including an optimized dPCR master mix, the PanCancer Kits achieve exceptional sensitivity and specificity. This allows for the detection of multiple mutations in a single channel at allelic frequencies below 0.5%. When combined with QIAGEN's solutions for DNA extraction, a diverse range of sample types - such as blood, plasma, FFPE, or stool samples - can be analyzed for the presence of hallmark mutations.

Principle and procedure

The dPCR PanCancer Kits consist of two boxes: one containing the QIAcuity masterMix and a second box containing the PanCancer Assay plus the reference assay. Both are designed to be used exclusively with the dedicated QIAcuity masterMix of the kit. The assays are supposed to be run on a 26K 24-well nanoplate but can also be used together with other QIAcuity nanoplate types.

Various DNA sample preparation products from QIAGEN, coupled with the QIAcuity instrument, offer unique sample-to-insight solutions for quantifying hallmark mutations in BRAF and EGFR. This sample-to-insight workflow involves a straightforward procedure with up to three steps, including (1) the extraction of DNA from various sample types, (2) the preparation of the dPCR reaction mix, and (3) quantification of hallmark mutations in dPCR (see Figure 1).

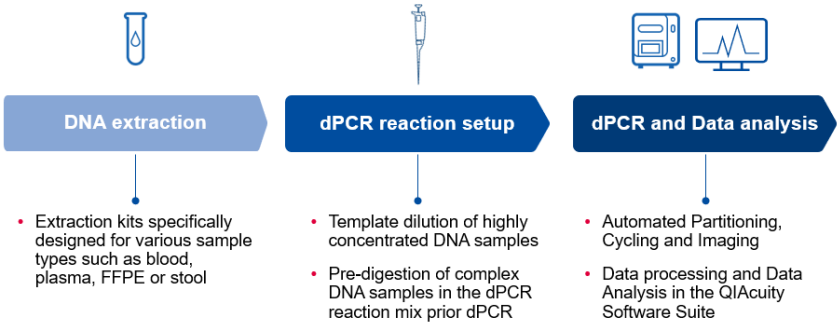

Figure 1. Simplified overview of the workflow.

DNA Extraction

For optimal yield and quality of the DNA, several sample-specific parameters should be considered for the selection of the DNA extraction kit. This includes, for example the sample type, the sample quality, the sample volume, the DNA yield, the content of inhibitors or the compatibility with automated workflows. QIAGEN provides a diverse range of DNA extraction kits and technologies optimized for various sample types, including blood, plasma, or FFPE samples. Recommended protocols for the manual and automated extraction of DNA from blood and plasma can be found in the Protocols section below. Additional recommendations for extraction kits are listed in Table 2.

Table 2. Recommended DNA extraction kits for diverse sample types

Sample Type	Recommended DNA Extraction Kit(s)	Catalog Numbers
Blood	QIAamp® Blood Kits Mini	51104
	EZ1&2™ Blood 350 µL Kit	951054
	QIAsymphony® DSP DNA Midi Kit (96)	937255, 937236
Plasma	QIAamp circulating nucleic acid kit (CNA)	55114
	QIAamp MinElute ccfDNA midi	55284, 55204
	EZ1&2 ccfDNA Kit	954854
	QIAsymphony PAXgene® Blood ccfDNA Kit	768536
	QIAsymphony DSP circulating DNA Kit	937556
Fresh Tissue	QIAamp Fast DNA Tissue Kit	51404
	EZ1&2 DNA Tissue Kit	952034
FFPE Tissue	QIAamp DNA FFPE Advanced Kit	56604
	EZ1&2 DNA FFPE	954404
	QIAsymphony DSP DNA Kit (Mini)	937255, 937236

Template Dilution

A template dilution is necessary for highly concentrated DNA samples to prevent overloading of the nanoplate well. The PanCancer assays have undergone wet lab testing for input amounts of up to 50 ng of human DNA. Although higher input volumes of up to 250 ng have been tested, in some cases, they may exhibit reduced sensitivity.

Template Digestion

In the case of complex genomic DNA, a digestion of the input DNA is necessary to ensure the random distribution of templates across the well, a crucial requirement for accurate quantification in dPCR systems. When using PCR products, formalin-fixed, paraffin-embedded (FFPE) DNA, circulating cell-free DNA (cfDNA), or complementary DNA (cDNA) as templates, a uniform distribution of PCR signal is observed and no digestion has to be done.

However, DNA molecules larger than 30 kb exhibit uneven partitioning, leading to the overquantification of template concentration. In such cases, restriction enzymes must be directly added to the QIAcuity reaction mixes to fragment larger DNA molecules into smaller sizes, resulting in an even template distribution and accurate quantification. The restriction enzymes recommended for the PanCancer Assays are carefully selected to avoid cutting within the amplicon sequences of the different mutations and wild-type (WT) templates.

PanCancer Assays and Reference Assay AP3B1

The assays are supplied as a ready-to-use 20x primer-probe mix. The PanCancer assay is labeled with FAM, and the reference assay AP3B1 is labeled with HEX. Both assays are simultaneously run in a duplex reaction, where the PanCancer assay selectively identifies templates carrying any of the listed mutations (refer to Table 1), while the reference assay detects and quantifies the human AP3B1 gene, representing the genome copy number in the sample.

Equipment and Reagents to be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Restriction enzyme BsuRI (HaeIII) (tested product from Thermo Scientific™, 10 U/µL, cat. no. ER0151).
- QIAcuity Nanoplates (cat. nos. 250001, 250011, or 250021)
- Microcentrifuge tubes or PCR plates or strip tubes with appropriate sealing foil. Compatibility of tubes/plates with heating devices needed
- Single-channel or multichannel pipettor (manual or automatic) with nuclease-free, aerosol-barrier pipette tips
- Vortexer
- Centrifuge for tubes and plates

Important Notes

Quantification and Detection of low-abundance mutations

The detection of low-abundance mutations, or low mutation frequencies is crucial for an early detection and a better understanding of disease progression. Some sample types are either low concentrated such as ccfDNA or are limited material such as tumor biopsies. Detection of low-abundance mutations in these samples is limited by the number of mutation templates that can be added to the dPCR reaction. The 26K nanoplate can be loaded with up to 26 µL of input template enabling a higher sensitivity compared to other dPCR platforms.

In order to detect a target in the dPCR reaction at least one target molecule has to end up in one partition of the nanoplate well. For statistical robustness at least 10 target molecules are required in the 40 µL dPCR reaction of the 26K nanoplate well to pick up the mutation with a single dPCR reaction with a confidence interval of 95%. This sets a lower limit of target molecules in the dPCR that can be detected. At a mutation frequency of 0.1% these 10 mutation copies represent a total input of ca. 33 ng of human DNA into the dPCR reaction considering that 3.3 pg represents the genomic content of a single copy of the human genome.

> **Important**: The 2nd level analysis tool for mutation frequencies within the QIAcuity software suite cannot be used for the PanCancer assay results because it employs a distinct calculation metric specific for the LNA Mutation assays (cat. no. 250200). Further details see in the section “Calculation of mutation frequencies” below.

Limit of Blank (LOB) and Limit of Detection (LOD)

The limit of blank and the limit of detection are crucial parameters for interpreting dPCR results regarding the presence or absence of a mutation. The NTC method enhances detection sensitivity by measuring assay background, expressed as the limit of blank (LOB). Correcting for assay background and/or contamination from environmental factors involves conducting a dPCR reaction with No Template Control (NTC) as the sample. NTC establishes a threshold for the number of positive partitions, above which a target in the sample is considered positively detected; this threshold is referred to as the limit of detection (LOD) and may vary across assays and sample types. As illustrated in Table 3, the number of detected copies/µL in three NTC and three sample replicates is provided.

The LOD is calculated as follows*:

- Average number of measured copies/µL in NTC ($mean^{NTC}$) = (0+0.055+0)/3=0.018
- Standard deviation of measured copies/µL in NTC (SD^{NTC}) = 0.026
- Limit of Blank (LOB) = meanNTC +1.645*(SD^{NTC}) = 0.018+1.645×0.026 = 0.061
- Average number of measured copies/µL in Sample ($mean^{Sample}$) = (0.268+0.386+0.377)/3 = 0.344
- Standard deviation of measured copies/µL in Sample (SD^{Sample}) = 0.054
- Limit of Detection (LOD) = LOB +1.645*(SD^{Sample}) = 0.061+1.645×0.054 = 0.149 copies/µL
- LOD in total copies per reaction (8.5K nanoplate) = LOD × 12 = 0.149×12 = 1.79 copies
- LOD in total copies per reaction (26K nanoplate) = LOD × 40 = 0.149×40 = 5.96 copies
    - For this particular example the target mutation can be considered as present as the on average measured copies/µL value ($mean^{Sample}$) is >0.149

* Armbruster, D. A., & Pry, T. (2008). Limit of blank, limit of detection and limit of quantitation. *The clinical biochemist reviews*, 29(Suppl 1), S49.

- For cases of ($mean^{Sample}$) < LOD the mutation should be considered absent for the target.

Table 3. Example of detected positive partitions for 3 NTC and 3 Sample replicates

Replicate	Measured Copies/µL
NTC Replicate 1	0
NTC Replicate 2	0.055
NTC Replicate 3	0
Sample Replicate 1	0.268
Sample Replicate 2	0.386
Sample Replicate 3	0.377

Protocol: Manual Extraction of gDNA from Blood Using the QIAamp DNA Blood Mini Kit

This protocol is for purification of genomic DNA from 200 µL of human blood. Additional protocols for the extraction from 350 µL of human blood can be found at QIAamp DNA Blood Kits product page (**www.qiagen.com/QIAampDNABloodKits**) and *QIAamp DNA Mini Blood Mini Handbook* (**www.qiagen.com/HB-0329**).

Important points before starting

- Perform all centrifugation steps at room temperature (15–25°C).
- Dissolve any precipitates in Buffer AL by warming at 56°C until the precipitate has dissolved.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates, as indicated on the bottle.
- Add Protease Solvent to lyophilized QIAGEN Protease, as indicated on the label.
- Equilibrate samples to room temperature (15–25°C).
- Preheat a water bath or heating block to 56°C.

Further Information

- *QIAamp DNA Mini and Blood Mini Handbook*: **www.qiagen.com/HB-0329**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Procedure

1. Pipette 20 µL QIAGEN Protease into a 1.5 mL microcentrifuge tube. Add 200 µL sample. If the sample volume is less than 200 µL, add the appropriate volume of PBS.
2. Add 200 µL Buffer AL. Mix thoroughly by pulse-vortexing for 15 s.

3. Incubate at 56°C for 10 min. Briefly centrifuge the 1.5 mL microcentrifuge tube to remove drops from the lid.
4. Add 200 µL ethanol (96–100%). Mix thoroughly by vortexing. Briefly centrifuge the tube to remove drops from the lid.
5. Pipette the mixture onto the QIAamp Mini spin column (in a 2 mL collection tube) and centrifuge at 6000 x *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.

   Note: When preparing DNA from buffy coat or lymphocytes, centrifugation at full speed is recommended to avoid clogging.
6. Place the QIAamp Mini spin column in a new 2 mL collection tube and add 500 µL Buffer AW1. Centrifuge at 6000 x *g* (8000 rpm) for 1 min. Discard the flow-through and collection tube.
7. Place the QIAamp Mini spin column in a new 2 mL collection tube and add 500 µL Buffer AW2. Centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min. Discard the flow-through and collection tube.
8. Place the QIAamp Mini spin column in a new 2 mL collection tube (not provided) and centrifuge at full speed for 1 min. This eliminates a carryover of Buffer AW2.
9. Place the QIAamp Mini spin column in a new 1.5 mL microcentrifuge tube (not provided), add 200 µL Buffer AE or distilled water, and incubate at room temperature (15–25°C) for 5 min. Centrifuge at 6000 x *g* (8000 rpm) for 1 min to elute the DNA.

Protocol: Automated Extraction of gDNA from Blood Using the EZ1&2 Blood 350 µL Kit with the EZ2®

This protocol is for purification of genomic DNA from 350 µL of human blood. Additional protocols for the extraction from 200 µL of human blood can be found at EZ1&2 Blood Kits product page (**www.qiagen.com/E1&EZ2BloodKits**) and *EZ1&2 DNA Blood Handbook* (**www.qiagen.com/HB-0197**).

Important notes before starting

- To use the EZ1&2 DNA Blood 350 µL Kit with the EZ1® instrument, refer to the corresponding handbook (**www.qiagen.com/HB-0197**) and quick-start protocol (**www.qiagen.com/HB-0782**)
- The buffer in well 1 of the reagent cartridge may form a precipitate upon storage. If necessary, re-dissolve by warming at 37°C and then place at room temperature.
- EZ1 instruments should only be switched on after an EZ1 Card is inserted. Make sure that the EZ1 Card is completely inserted, otherwise essential instrument data could be lost. EZ1 Cards should not be exchanged while the instrument is switched on.
- Before loading reagent cartridges into the EZ1 instrument, invert the cartridges 4 times to mix the magnetic particles and then tap to deposit the reagents at the well bottoms. Check that the magnetic particles are completely resuspended.

Further Information

- *EZ1&2 DNA Blood Handbook for use with EZ2 Connect instruments*: **www.qiagen.com/HB-2965**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Procedure

1. Switch on the EZ2 Connect instrument.
2. Tap **DNA** on the Applications panel and then select the **DNA Blood 200 µL Kit** and press **Next**.
3. Choose the DNA Blood protocol and press **Next**.
4. Set sample volume to "350 µL" and elution volume to "100 µL". Select if wash step with ethanol (optional) should be performed and press **Next**.
5. Select positions on the work deck according to the number of samples to be processed and press **Next**.
6. Enter sample IDs or press **Generate missing sample IDs**. Then press **Next**.
7. Gently invert reagent cartridges 4 times to mix the magnetic particles. Tap the cartridges to deposit the reagents at the bottom of their wells. Check that the magnetic particles are completely resuspended.
8. Load the EZ1&2 Blood reagent cartridges into the positions of the EZ2 Connect Cartridge Rack that were selected in step 5.
9. Open the instrument hood. Load the EZ2 Connect Cartridge Rack into the EZ2 Connect instrument.

   9a. Remove caps of all tubes and prepare the EZ2 Connect Tip Rack as follows:
   - Position A: 2.0 mL sample tube
   - Position B: 2.0 mL tube with 1800 µL 80% ethanol (optional, see step 4)
   - Position C: Tip holder with Filter Tips
   - Position D: 1.5 mL empty elution tube

   9b. Press **Next**.
10. Place the EZ2 Connect Tip Rack into the EZ2 Connect instrument and start the run according to the instructions on the instrument display.

11. The display will show “Protocol finished” when the run is completed. Select **Finish**.
12. Open the instrument hood. Remove the elution tube containing purified nucleic acid from position D of the EZ2 Connect Tip Rack. Discard the used cartridge including the liquid waste.
13. Perform regular maintenance after each run. Press **Finish** to return to the home screen.

Protocol: Automated Extraction of gDNA from Blood Using the QIAsymphony DSP DNA Midi Kit with the QIAsymphony

This protocol is for purification of genomic DNA from 1000 µL of human blood. Additional protocols for the extraction from lower and higher volumes of human blood can be found at product page (**www.qiagen.com/QIAsymphonyDSPDNAKits**) and *QIAsymphony DSP DNA Kit Instructions for Use (Handbook)* (**www.qiagen.com/HB-3029**).

Important notes before starting

- Make sure that you are familiar with operating the QIAsymphony SP. Refer to the user manuals supplied with your instrument for operating instructions.
- Optional maintenance is not mandatory for instrument function but is highly recommended to reduce risk of contamination.
- Before beginning the procedure, read "Principle and procedure" starting on page 11.
- Make sure you are familiar with the protocol sheet corresponding to the procedure you want to use (**www.qiagen.com/HB-3029**).
- Before using a reagent cartridge for the first time, check that Buffers QSL1 and QSB1 do not contain any precipitates. If necessary, remove the troughs containing Buffers QSL1 and QSB1 from the reagent cartridge and incubate for 30 min at 37°C with occasional shaking to dissolve precipitates. Make sure to replace the troughs in the correct positions. If the reagent cartridge is already pierced, make sure that the troughs are sealed with Reuse Seal Strips and incubate the complete reagent cartridge for 30 minutes at 37°C with occasional shaking in a water bath.
- Try to avoid vigorous shaking of the reagent cartridge (RC) otherwise foam may be generated, which can lead to liquid-level detection problems

Further Information

- For information about sample tubes compatible with a certain protocol, see the corresponding labware list, which can be found under the resource tab of the product page on **www.qiagen.com/QIAsymphonyDSPDNAKits.**
- For information about minimum sample volumes for secondary tubes, see the corresponding labware list, which can be found under the resource tab of the product page on **www.qiagen.com/QIAsymphonyDSPDNAKits.**
- For more information, refer to *QIAsymphony® DSP DNA Midi Kit Instructions for Use (Protocol Sheet)* (**www.qiagen.com/HB-3029**).

Procedure

1. Close all drawers and the hood.
2. Power ON the QIAsymphony SP, and wait until the Sample Preparation screen appears and the initialization procedure has finished. The power switch is located at the bottom, left corner of the QIAsymphony SP.
3. Log on to the instrument.
4. Make sure that the "Waste" drawer is properly prepared and perform an inventory scan of the "Waste" drawer, including the tip chute and liquid waste. Replace the tip disposal bag if necessary.
5. Load the required elution rack into the "Eluate" drawer. Do not load a 96-well plate onto "Elution slot 4". "Elution slot 1", with the corresponding cooling adapter, must be used. When using a 96-well plate, make sure that the plate is in the correct orientation, as incorrect placement may cause sample mix-up in downstream analysis. When using the Elution Microtubes CL rack, remove the bottom by twisting the rack until the bottom comes off.
6. Load the required reagent cartridge(s) and consumables into the "Reagents and Consumables" drawer.
7. Perform an inventory scan of the "Reagents and Consumables" drawer.

8. Place the samples into the appropriate sample carrier, and load them into the “Sample” drawer.

   Note: To ensure correct liquid level detection, push the tubes down to the bottom of the tube carrier or insert, if inserts are used.

9. Using the touchscreen, enter the required information for each batch of samples to be processed. Enter the following information:

   9a. Sample information (depending on sample racks used)
   9b. Protocol to be run (Assay Control Set)
   9c. Elution volume of 400 µL and output position

10. Press the **Run** button to start the purification procedure. All processing steps are fully automated. At the end of the protocol run, the status of the batch changes from “RUNNING” to “COMPLETED”.
11. Retrieve the elution rack containing the purified nucleic acids from the “Eluate” drawer.
12. The DNA is ready to use or can be stored. Details are given in the relevant protocol sheets available at **www.qiagen.com/QIAsymphonyDSPDNAKits** We recommend removing the eluate plate from the “Eluate” drawer immediately after the run has finished. Depending on temperature and humidity, elution plates left in the QIAsymphony SP after the run is completed may experience condensation or evaporation. In general, magnetic particles are not carried over into eluates. If carryover does occur, magnetic particles in eluates will not affect most downstream applications. If magnetic particles need to be removed before performing downstream applications, tubes, or plates containing eluates should first be placed in a suitable magnetic rack and the eluates transferred to a clean tube. Result files are generated for each elution plate.
13. If a reagent cartridge is only partially used, seal it with the provided Reuse Seal Strips and close tubes containing Proteinase K with screw caps immediately after the end of the protocol run to avoid evaporation.
14. Discard used sample tubes and waste according to your local safety regulations.

15. Clean the QIAsymphony SP. Follow the maintenance instructions in the user manuals supplied with your instrument. Make sure to clean the tip guards regularly to minimize the risk of cross-contamination.
16. Close the instrument drawers and power OFF the QIAsymphony SP.

Protocol: Preparation of Plasma from Blood Samples

This protocol is for preparation of plasma from human blood.

Plasma preparation using PrepEDTA blood in BD Vacutainer® tubes

Important notes before starting

- Cool down the centrifuge to 4°C

Procedure

1. Place whole blood in a collection tube into a centrifuge with a swing-out rotor and appropriate buckets.
2. Centrifuge the blood samples for 10 min at 1900 x *g* with temperature set to 4°C.
3. Carefully aspirate plasma supernatant without disturbing the buffy coat layer. Approximately 4–5 mL plasma can be obtained from one 10 mL primary blood tube.

   Note: Plasma can be used for circulating nucleic acid extraction at this stage. However, the following high-speed centrifugation will remove additional cellular debris and contamination of the circulating nucleic acids by genomic DNA and RNA derived from damaged blood cells.
4. Transfer aspirated plasma into new 15 mL centrifuge tubes with conical bottoms.
5. Centrifuge the plasma samples for 15 min at 3000 x *g* in a fixed-angle rotor with temperature set to 4°C. This will remove additional cellular nucleic acids attached to cell debris.
6. Using a pipette, carefully transfer the supernatant into a new tube without disturbing the pellet.

7. If plasma will be used for nucleic acid extraction on the same day, store at 2–8°C until further processing. For longer storage, keep plasma frozen at –90°C to –65°C. Before using the plasma for circulating nucleic acid extraction, thaw plasma tubes at room temperature or at 37°C in a water bath.

Plasma preparation using PAXgene Blood ccfDNA Tube

To isolate circulating, cell-free nucleic acids from blood samples, we recommend following this protocol, which includes a high g-force centrifugation step to remove cellular debris and reduce the amount of cellular or genomic DNA and RNA in the sample. Human serum or plasma samples can be generated using blood collection tubes such as the PAXgene Blood ccfDNA Tube; please refer to the manufacturer´s recommendation for plasma separation procedure (see **www.qiagen.com/PAXgeneBloodccfDNATubes100**) and *PAXgene Blood ccfDNA Tube (RUO) Handbook* (**www.qiagen.com/PROM-1710**).

Procedure

1. Place whole blood in a collection tube into a centrifuge with a swing-out rotor and appropriate buckets.
2. Centrifuge the blood samples for 15 min at 1900 x *g* at room temperature (15–25°C).
3. Carefully aspirate plasma supernatant without disturbing the buffy coat layer. Approximately 4–5 mL plasma can be obtained from one 10 mL primary blood tube.

   Note: Plasma can be used for circulating nucleic acid extraction at this stage. However, the following high-speed centrifugation will remove additional cellular debris and contamination of the circulating nucleic acids by genomic DNA and RNA derived from damaged blood cells.
4. Transfer aspirated plasma into new 15 mL centrifuge tubes with conical bottoms.
5. Centrifuge the plasma samples for 10 min at 3000 x *g* in a fixed-angle rotor with temperature set with room temperature (15–25°C). This will remove additional cellular nucleic acids attached to cell debris.

6. Using a pipette, carefully transfer the supernatant into a new tube without disturbing the pellet.
7. If plasma will be used for nucleic acid extraction on the same day, store at room temperature (15–25°C) until further processing. For longer storage, keep plasma frozen at –90°C to –65°C. Before using the plasma for circulating nucleic acid extraction, thaw plasma tubes at room temperature or at 37°C in a water bath.

Protocol: Manual Extraction of ccfDNA from Plasma Using the QIAamp Circulating Nucleic Acid Kit

This protocol is for purification of circulating DNA from 4 mL plasma. Additional protocols for the extraction from 1 mL, 2 mL, 3 mL, and 5 mL plasma, serum or urine can be found at QIAamp Circulating Nucleic Acid Kit product page (**www.qiagen.com/QIAampCirculatingNucleicAcidKit**) and *QIAamp Circulating Nucleic Acid Handbook* (**www.qiagen.com/HB-0202**).

Important notes before starting

- All centrifugation steps are carried out at room temperature.
- Switch off vacuum between steps to ensure that a consistent, even vacuum is applied during protocol steps.
- Plasma samples should be at room temperature before use. If plasma/serum was stored frozen, thaw the samples by incubating at 37°C in a water bath.

Things to do before starting

- Equilibrate samples to room temperature. If samples are <4 mL, bring the volumes up to 4 mL with phosphate-buffered saline.
- Set up the QIAvac 24 Plus as described in the *QIAvac 24 Plus Handbook* (**www.qiagen.com/HB-0496**).
- Heat a water bath or heating block to 60°C for use with 50 mL centrifuge tubes in step 4.
- Heat a heating block to 56°C for use with 2 mL collection tubes in step 14.
- Equilibrate Buffer AVE to room temperature for elution in step 15.

- Ensure that Buffer ACB, Buffer ACW1 and Buffer ACW2 have been prepared according to the instructions in "Important Notes".
- Add carrier RNA reconstituted in Buffer AVE to Buffer ACL according to instructions in Table 4.

Table 4. Volumes of Buffer ACL and carrier RNA (dissolved in Buffer AVE) required for processing 4 mL samples

Number of samples	Buffer ACL (mL) for processing 4 mL	Carrier RNA in Buffer AVE (µL)
1	3.5	5.6
2	7.0	11.3
3	10.6	16.9
4	14.1	22.5
5	17.6	28.1
6	21.1	33.8
7	24.6	39.4
8	28.2	45.0
9	31.7	50.6
10	35.2	56.3
11	38.7	61.9
12	42.2	67.5
13	45.8	73.1
14	49.3	78.8
15	52.8	84.4
16	56.3	90.0
17	59.8	95.6
18	63.4	101.3
19	66.9	106.9
20	70.4	112.5
21	73.9	118.1
22	77.4	123.8
23	81.0	129.4
24	84.5	135.0

Procedure

1. Pipette 400 µL QIAGEN Proteinase K into a 50 mL centrifuge tube (not provided).
2. Add 4 mL of plasma to the tube.
3. Add 3.2 mL Buffer ACL (containing 1.0 µg carrier RNA). Close the cap and mix by pulse-vortexing for 30 s.

   Make sure that a visible vortex forms in the tube. To ensure efficient lysis, it is essential that the sample and Buffer ACL are mixed thoroughly to yield a homogeneous solution.

   Note: Do not interrupt the procedure at this time. Proceed immediately to step 4 to start the lysis incubation.
4. Incubate at 60°C for 30 min.
5. Place the tube back on the lab bench and unscrew the cap.
6. Add 7.2 mL Buffer ACB to the lysate in the tube. Close the cap and mix thoroughly by pulse-vortexing for 15–30 s.
7. Incubate the lysate–Buffer ACB mixture in the tube for 5 min on ice.
8. Insert the QIAamp Mini column into the VacConnector on the QIAvac 24 Plus. Insert a 20 mL tube extender into the open QIAamp Mini column.

   Make sure that the tube extender is firmly inserted into the QIAamp Mini column to avoid leakage of sample.

   Note: Keep the collection tube for the dry spin in step 13.
9. Carefully apply the lysate–Buffer ACB mixture from step 7 into the tube extender of the QIAamp Mini column. Switch on the vacuum pump. When all lysates have been drawn through the columns completely, switch off the vacuum pump and release the pressure to 0 mbar. Carefully remove and discard the tube extender.

   Please note that large sample lysate volumes may need up to 15 min to pass through the QIAamp Mini membrane by vacuum force. For fast and convenient release of the

vacuum pressure, the Vacuum Regulator should be used (part of the QIAvac Connecting System).

Note: To avoid cross-contamination, be careful not to move the tube extenders over neighboring QIAamp Mini Columns.

10. Apply 600 µL Buffer ACW1 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW1 has been drawn through the QIAamp Mini column, switch off the vacuum pump, and release the pressure to 0 mbar.
11. Apply 750 µL Buffer ACW2 to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of Buffer ACW2 has been drawn through the QIAamp Mini column, switch off the vacuum pump, and release the pressure to 0 mbar.
12. Apply 750 µL of ethanol (96–100%) to the QIAamp Mini column. Leave the lid of the column open, and switch on the vacuum pump. After all of ethanol has been drawn through the spin column, switch off the vacuum pump, and release pressure to 0 mbar.
13. Close the lid of the QIAamp Mini column. Remove it from the vacuum manifold, and discard the VacConnector. Place the QIAamp Mini column in a clean 2 ml collection tube, and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.
14. Place the QIAamp Mini Column into a new 2 mL collection tube. Open the lid and incubate the assembly at 56°C for 10 min to dry the membrane completely.
15. Place the QIAamp Mini column in a clean 1.5 mL elution tube (provided) and discard the 2 mL collection tube from step 14. Carefully apply 60 µL (possible range) of Buffer AVE to center of the QIAamp Mini membrane. Close the lid and incubate at room temperature for 3 min.

    Important: Ensure that the elution buffer AVE is equilibrated to room temperature. If elution is done in small volumes (<50 µL) the elution buffer has to be dispensed onto the center of the membrane for complete elution of bound DNA.

Elution volume is flexible (20–150 µL) and can be adapted according to the requirements of downstream applications. The recovered eluate volume will be up to 5 µL less than the elution volume applied to the QIAamp Mini column.

16. Centrifuge in a microcentrifuge at full speed (20,000 x *g* ;14,000 rpm) for 1 min to elute the nucleic acids.

Protocol: Automated Extraction of ccfDNA from Plasma Using the EZ1&2 ccfDNA Kit with the EZ2

This protocol is for purification of circulating DNA from 8 mL plasma. Additional protocols for the extraction from lower volumes of plasma, serum or urine can be found at EZ1&2 ccfDNA Kit product page (**www.qiagen.com/EZ1&2ccfDNAKit**) and *EZ1&2 ccfDNA Kit Handbook* (**www.qiagen.com/HB-2915**). The EZ1&2 ccfDNA Kit has been optimized for sample volumes of 1–8 mL.

Important notes before starting

- Plasma samples should be at room temperature before use. If plasma/serum was stored frozen, thaw the samples by incubating at 37°C in a water bath.
- If the sample volume is less than 8 mL, adjust the volume with PBS to 8 mL.

Procedure

1. Load reagent cartridges into the cartridge rack.
2. Vortex the Magnetic Bead Suspension EZ briefly in an upright position.
3. Turn the tube with the cap facing downwards and vortex again to ensure a homogeneous bead suspension.
4. Briefly centrifuge to collect all the liquid at the bottom of the tube. Remove the cap from the tube with the bead solution and place in position 11 of the EZ1&2 ccfDNA cartridge.

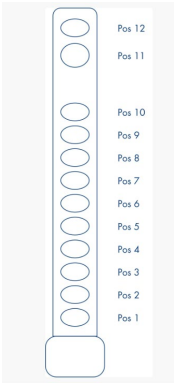

Figure 2. EZ1&2 ccfDNA cartridge.

5. Transfer 900 µL Elution Buffer EZE into position 12 of the EZ1&2 ccfDNA cartridge.
6. Remove caps of all tubes and prepare the Tip Rack as follows (see Figure 3):
   - Position 4/A: Tip holder with Filter Tip (provided)
   - Position 3/B: new large volume tube (7 mL) (provided)
   - Position 2/C: new large volume tube (7 mL) (provided)
   - Position 1/D: new 1.5 mL elution tube (provided)

Figure 3. Tip Rack - Large Volume.

7. Eight milliliter protocol: Split your sample and transfer 4 mL into each of the large volume tubes in positions 2/C and 3/B.

   Note: Procedure for the EZ1® Advanced XL is given in the extraction kit *EZ1 Advanced XL User Manual* (**www.qiagen.com/HB-0176**).

8. Turn on the EZ2 Connect instrument.
9. Tap **DNA** on the Applications panel and select the **EZ1&2 ccfDNA Kit** and press **Next**. Follow onscreen instructions for selection of protocol, parameter definition, sample position selection, sample IDs, and worktable setup.
10. Open the instrument door. Load the cartridge rack into the instrument.
11. Place the tip rack into the instrument.
12. Close the instrument door; press **Start** to initiate the EZ1&2 ccfDNA protocol.
13. The display will show "Protocol finished" when the run is completed. Select **Finish**.

    Open the instrument hood. Remove the elution tubes containing the purified ccfDNA from position 1/D of the tip rack. The eluate volume can range from 60–75 µL . Discard the sample preparation waste (in tubes in positions 2/C and 3/B and in case of the 8 mL protocol in position 4/A) (see Figure 3).

    Optional: Follow onscreen instructions for UV decontamination of worktable surfaces.
14. Perform regular maintenance after each run. Press **Finish** to return to the Home Screen.

Protocol: Automated Extraction of ccfDNA from Plasma Using the QIAsymphony DSP Circulating DNA Kit with the QIAsymphony

This protocol is for purification of circulating DNA from 4 mL plasma. Additional protocols for the extraction from 2 mL of plasma or urine can be found at QIAsymphony DSP Circulating DNA Kit product page (**www.qiagen.com/QIAsymphonyDSPCirculatingDNAKit**) and *QIAsymphony DSP Circulating DNA Kit Handbook* (**www.qiagen.com/HB-3029**).

Important notes before starting

- Make sure that you are familiar with operating the QIAsymphony SP. Refer to the user manuals supplied with your instrument for operating instructions.
- Optional maintenance is not mandatory for instrument function but is highly recommended to reduce risk of contamination.
- Make sure that you are familiar with the protocol sheet corresponding to the procedure you want to use (see **www.qiagen.com/HB-3034**).
- Avoid vigorous shaking of the reagent cartridge otherwise foam may be generated, which can lead to liquid-level detection problems.
- Before starting a pretreatment that requires Buffer ATL, check whether precipitate has formed in Buffer ATL. If necessary, dissolve precipitate by heating at 70°C with gentle agitation in a water bath. Aspirate bubbles from the surface of Buffer ATL.
- Plasma samples should be at room temperature before use. If plasma/serum was stored frozen, thaw the samples by incubating at 37°C in a water bath.

Things to do before starting

- Before starting the procedure, make sure that the magnetic particles are fully resuspended. Vortex the trough containing the magnetic particles vigorously for at least 3 min before first use.
- Make sure that the piercing lid is placed on the reagent cartridge and the lid of the magnetic-particle trough has been removed or, if using a partially used reagent cartridge, make sure the Reuse Seal Strips have been removed.
- Proteinase K is not included in the reagent cartridge but has to be provided by the user (sample drawer, slot A, position 1, 2, and/or 3). Make sure that correct Proteinase K volume is available. For detailed information, see Q*IAsymphony DSP Circulating DNA Kit Instructions for Use Protocol Sheet* (**www.qiagen.com/HB-3034**).
- If samples are bar coded, orient samples in the tube carrier so that the bar codes face the bar code reader at the left side of the QIAsymphony SP.
- For information about sample tubes compatible with a certain protocol, see the corresponding labware list, which can be found under the resource tab of QIAsymphony DSP Circulating DNA Kit product page (**www.qiagen.com/QIAsymphonyDSPCirculating DNAKit**).
- For information about minimum sample volumes for secondary tubes, see the corresponding labware list, which can be found under the resource tab of the product page (**www.qiagen.com/QIAsymphonyDSPCirculating DNAKit**).
- For more information , refer to the *QIAsymphony DSP Circulating DNA Kit Instructions for Use Protocol Sheet* (**www.qiagen.com/HB-3034**).

Procedure

1. Close all drawers and the hood.
2. Power ON the QIAsymphony SP, and wait until the Sample Preparation screen appears and the initialization procedure has finished. The power switch is located at the bottom, left corner of the QIAsymphony SP.

3. Log on to the instrument.
4. Load the required elution rack into the “Eluate” drawer. Do not load a 96-well plate onto “Elution slot 4”. “Elution slot 1”, with the corresponding cooling adapter, must be used. When using a 96-well plate, make sure that the plate is in the correct orientation, as incorrect placement may cause sample mix-up in downstream analysis. When using the Elution Microtubes CL rack, remove the bottom by twisting the rack until the bottom comes off.
5. Make sure that the “Waste” drawer is properly prepared by performing an inventory scan of the “Waste” drawer, including the tip chute and liquid waste. Replace the tip disposal bag if necessary.
6. Load the required reagent cartridge(s) and consumables into the “Reagents and Consumables” drawer.
7. Perform an inventory scan of the “Reagents and Consumables” drawer.
8. Place the samples into the appropriate sample carrier, and load them into the “Sample” drawer.
9. Using the touchscreen, enter the required information for each batch of samples and for proteinase K to be processed.

   Enter the following information:

   - Sample information (depending on sample racks used)
   - Protocol to be run (ACS_circDNA_4000_DSP_V2)
   - Elution volume of 60 µL and output position

   After information about the batch has been entered, the status changes from LOADED to QUEUED. As soon as one batch is queued, the Run button appears.
10. Tubes containing proteinase K are placed in a tube carrier. For each sample of 4 mL plasma, 220 µL proteinase K are required, plus an additional void volume of 1100 µL [(n x 220 µL) + 1100 µL]. The tube(s) containing the proteinase K must be placed on positions 1, 2, and/or 3 in slot A of the “Sample” drawer. The QIAsymphony DSP

Circulating DNA Kit contains ready-to-use proteinase K solution that can be stored at room temperature.

11. Define the proteinase K by pressing the IC button.
12. Press the Run button to start the purification procedure. All processing steps are fully automated. At the end of the protocol run, the status of the batch changes from RUNNING to COMPLETED.
13. Retrieve the elution rack containing the purified nucleic acids from the "Eluate" drawer.
14. The DNA is ready to use or can be stored at 2–8°C, –20°C, or –80°C.

    We recommend removing the eluate plate from the "Eluate" drawer immediately after the run has finished. Depending on temperature and humidity, elution plates left in the QIAsymphony SP after the run is completed may experience condensation or evaporation.

    In general, magnetic particles are not carried over into eluates. If carryover does occur, magnetic particles in eluates will not affect most downstream applications.

    If magnetic particles need to be removed before performing downstream applications, tubes or plates containing eluates should first be placed in a suitable magnet and the eluates transferred to a clean tube. For further information see "Troubleshooting Guide" section in *QIAsymphony DSP Circulating DNA Kit Instructions for Use (Handbook)* (**www.qiagen.com/HB-3029**).

    Result files are generated for each elution plate.

15. If a reagent cartridge is only partially used, seal it with the provided Reuse Seal Strips after the end of the protocol run to avoid evaporation.

    Note: For more information about storage of partially used reagent cartridges, see "Reagent Storage and Handling" section in *QIAsymphony DSP Circulating DNA Kit Instructions for Use (Handbook)* (**www.qiagen.com/HB-3029**).

16. Discard used sample tubes and waste according to your local safety regulations.

17. Clean the QIAsymphony SP. Follow the maintenance instructions in the user manuals supplied with your instrument. Make sure to clean the tip guards regularly to minimize the risk of cross-contamination.
18. Close the instrument drawers and power off the QIAsymphony SP.

Protocol: Absolute Quantification of Multiple mutations using the dPCR PanCancer Assays

This protocol is optimized for the detection of multiple mutations in the specified target gene using the dPCR PanCancer Kits for BRAF V600 and EGFR exon19 deletions (cat. no. 250284, 250287) using QIAGEN's QIAcuity instruments for digital PCR (dPCR). Each kit consists of 2 boxes, one with the PanCancer Assay and one with the QIAcuity MasterMix.

The dPCR PanCancer Kit reagents should be stored immediately upon receipt at –30°C to –15°C in a constant-temperature freezer and protected from light. Unless otherwise indicated on the label, the components are stable until the expiration date indicated on the kit without showing any reduction in performance under these conditions.

Dedicated protocols for the various types of QIAGEN's QIAcuity dPCR assays can be found in their respective quick-start protocols and the *QIAcuity User Manual Extension: Application Guide*.

Further Information

- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**
- *QIAcuity User Manual Extension: Application Guide*: **www.qiagen.com/HB-2839**

Notes before starting

- Set up all reaction mixtures in an area separate from that used for DNA isolation and
- PCR product analysis (post-PCR).
- Use disposable tips containing hydrophobic filters to minimize cross-contamination.

- Always use the cycling conditions specified in the protocol. The cycling conditions have been optimized for this assay.
- Dilutions of DNA quantification standards in 1x TE buffer (pH8.0) can be stored at 4°C for at least 1 week.

Template DNA digestion

DNA samples with ⩾30 kb average length (e.g., genomic DNA purified via spin column with silica membrane, or salting out method) should be fragmented by restriction digestion before partitioning. Enzymatic fragmentation of larger DNA ensures even distribution of template throughout the partitions of the QIAcuity Nanoplate well, which in turn leads to accurate and precise quantification.

Restriction digestion is not required for highly fragmented DNA, e.g., FFPE DNA or circulating cell free DNA (ccfDNA).

Care should be taken to use enzymes that will not cut within the amplified sequence. For QIAGEN's PanCancer Assays digestion with the 4-cutter BsuRI (HaeIII) from Thermo Fisher Scientific is recommended. This validated enzyme will digest DNA in 10 min at room temperature (15–25°C) when added directly to the QIAcuity reaction mix at a concentration of 0.025 U/µL.

Procedure

1. Thaw the QIAcuity MasterMix, template DNA, PanCancer Assay, reference assay, and RNAse-free water. Vigorously mix the QIAcuity MasterMix and the individual solutions. Centrifuge briefly to collect liquids at the bottom of the tubes.
2. Prepare a reaction mix for the number of reactions needed according to Table 5. Due to the hot-start capability, it is not necessary to keep samples on ice during reaction setup or while programming the QIAcuity instrument.

Table 5. Reaction setup

	Volume/reaction	
Component	**Nanoplate 26k (24-well)**	**Final concentration**
4x QIAcuity MasterMix	10 µL	1x
20x PanCancer Assay	2 µL	1x
20x Reference Assay AP3B1	2 µL	1x
Restriction Enzyme (optional)	Up to 1 µL	0.025 U/µL
RNAse-free water	Variable	
Template DNA (added at step 5)	Variable*	
Total reaction volume	**40 µL**	

* Appropriate template amount depends on various parameters. Please see the *QIAcuity User Manual Extension: Application Guide for details*.

Note: Prepare a volume of master mix 10% greater than that required for the total number of PCR assays to be performed. This should include positive and negative control reactions.

3. Mix the master mix thoroughly and briefly centrifuge to collect the sample at the bottom of the tube.
4. Dispense appropriate volumes of the reaction mix, which contains all components except the template, into the wells of a standard PCR plate.
5. Then add template DNA into each well that contains the reaction mix and mix thoroughly and briefly centrifuge to collect sample at the bottom of the tube.

   Note: Proper mixing is crucial at this step to ensure homogenous distribution of template molecules in the reaction mix.
6. Transfer the content of each well from the standard PCR plate to the wells of the nanoplate.
7. Seal the nanoplate properly using the QIAcuity Nanoplate Seal provided in the QIAcuity Nanoplate Kits.

Note: For exact sealing procedure, please see the *QIAcuity System User Manual*.

8. If a restriction enzyme for DNA digestion has been included in the reaction, leave the plate for 10 min at room temperature.
9. Program the cycler of the QIAcuity instrument according to Table 6 and apply the imaging setting according to Table 7.

Table 6. Thermal cycling conditions

Step	Time	Temperature (°C)
PCR initial heat activation	2 min	95
2-step cycling (35 cycles*)		
Denaturation	15 s	95
Combined annealing/extension	20 s	55

* Number of cycles might vary depending on sample type. Additional 5 cycles might increase signal to noise separation.

Table 7. Imaging settings*

Channel	Exposure	Gain
Green (FAM)	500 ms	6
Yellow (HEX)	500 ms	6

* Imaging settings might need to be adjusted. Always start with the recommended settings.

10. Place the nanoplate into the QIAcuity instrument and start the dPCR program.

Data Analysis

1. To set up a plate layout according to the experimental design, open the QIAcuity Software Suite and define the reaction mixes, samples, and controls. Plate layout can be defined before or after the Nanoplate run.

   Note: Refer to the *QIAcuity User Manual* for details on setting up the plate layout.

2. After the run is completed, the raw data are automatically sent to the QIAcuity Software Suite.
3. For data analysis, open the QIAcuity Software Suite and select the individual Nanoplate for the analysis in Plate Overview of the Software Suite.

   Note: See the *QIAcuity User Manual Extension: Application Guide* and *QIAcuity User Manual* for details on how to analyze absolute quantification data.

Calculation of mutation frequencies

The mutation frequency in the analyzed sample is determined by calculating the ratio of the measured copies/µL from both the mutation assay and the reference assay AP3B1. The reference assay specifically targets the single-copy gene AP3B1, providing an accurate quantification of the total human genome copies in the sample, encompassing both mutated and wild-type copies. This quantification essentially represents the combined count of mutated and wild-type copies present. To compute the mutation frequency, simply divide the copies/µL obtained from the mutation assay by the corresponding copies/µL from the reference assay (AP3B1). This calculation method yields a reliable measure of the mutation frequency in the analyzed sample.

Important: The 2nd level analysis tool for mutation frequencies within the QIAcuity software suite employs a distinct calculation metric, making it unsuitable for calculating mutation frequencies for the PanCancer assays. This limitation arises from the tool's configuration, which calculates mutation frequency based on the quantification of target wildtype copies and target mutation copies, as specified by the LNA Mutation assay designs (cat. no. 250200).

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: **www.qiagen.com/FAQ/FAQList.aspx**. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, visit **www.qiagen.com**).

	Comments and suggestions
Handling and Storage	
Inconsistent results	• Make sure to mix reagents such as the 20x PanCancer Assay Mix or MasterMix before each use. Lack of homogeneity might lead to inconsistent results. • Avoid repeated freeze-thaw cycles of kit reagents. • Poor repeatability between different operators: Ensure operators are following the protocols. When using automatic pipettors, identical pipetting settings should be used by all operators (e.g., pre-dispense mode recommended over direct pipetting mode).
Storage	(Long-term) storage of intermediate workflow products not recommended
Pipetting volume	Ensure following lower and higher limit of your pipette. We recommend to not pipette volumes lower than 1 µL. Take viscosity of reagents into consideration and adjust pipetting setup accordingly.
Reference DNA	
Deviation from expected mutation frequencies	• Choice of restriction enzyme: If using other enzymes than those recommended in the protocols, make sure to test compatibility with the QIAcuity Mastermix contained in the PanCancer kits and the PanCancer Assay used. • Choice of reference DNA: Depending on the reference DNA mutation frequencies are adjusted for the specific mutations without adjustment of the genomic background. The PanCancer assays use the human AP3B1 housekeeping gene as a reference to quantify the genome copy numbers leading to underquantification of the total genome copy number in adjusted reference DNAs.

	Comments and suggestions
Assay performance	
Poor separation of positive and negative partitions (low delta RFU)	Insufficient amplification: Factors such as inhibitors, wrong cycling conditions, expired PCR and assay components can impact amplification efficiency that leads to lower end RFUs. Adding additional 5 cycles might boost the end RFU to enable a better signal to noise separation.
Rainy 1D scatterplots	• Multiple mutations: Depending on the mutation the end RFU of the PanCancer Assay can differ producing a broader end RFU spectrum in the 1D scatterplots. • Too much template input: The PanCancer assay are optimized for up to 50 ng of genomic DNA template input. Adding more can lead to individual partitions with reduced end RFU. Try increasing the cycles from 35 to 40. • Degraded template DNA: DNA isolated from samples such as FFPE tissue or plasma can contain heterogeneous template sequences with sequence alterations that can lead to divers amplification efficiencies in the first cycles of the dPCR reaction. Try to apply a manual threshold based on the threshold of the positive control.
No positive partitions	• Sample input below limit of detection (LOD). • Sample with low mutation frequency. Calculate expected number of mutation templates in the reaction based on the amount of input DNA and considering a single human genome copy of 3.3 pg. For reproducible detection of the target mutation in a single reaction at least 5 target copies should be added to the dPCR reaction in the 26K nanoplate. • Increase sample input into PCR. • Assays: Check compatibility with samples. • Imaging setup: Check channel choice and match with probe dyes. • Inhibition: Check for carryover of potential inhibitors in your samples from the upstream DNA extraction. Try using less sample input template and/or use extraction protocols optimized for removal of sample type-specific inhibitors.
No negative partitions	• Sample dilution: Increase dilution to fit into the dPCR concentration range. • Carryover of DNAse into the dPCR reaction.

Contact Information

For technical assistance and more information, please see our Technical Support Center at **www.qiagen.com/Support**, call 00800-22-44-6000, or contact one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit **www.qiagen.com**).

Ordering Information

Product	Contents	Cat. no.
BRAF PanCancer Kit BRAF V600 FAM (200)	20x BRAF V600 FAM assay, 20x AP3B1 HEX reference assay and 4x QIAcuity Master Mix for 200 reactions	250284
EGFR PanCancer Kit EGFRex19del FAM (200)	20x EGFR exon 19 FAM assay, 20x AP3B1 HEX reference assay and 4x QIAcuity Master Mix for 200 reactions	250287
QIAcuity Nanoplate 8.5K 96-well (10)	10 QIAcuity Nanoplates 8.5K with 96 wells, 11 Nanoplate Seals	250021
QIAcuity Nanoplate 8.5K 24-well (10)	10 QIAcuity Nanoplates 8.5K with 24 wells, 11 Nanoplate Seals	250011
QIAcuity Nanoplate 26K 24-well (10)	10 QIAcuity Nanoplates 26K with 24 wells, 11 Nanoplate Seals	250001
QIAcuity One, 2plex Instrument	One-plate digital PCR instrument for detecting up to 2 fluorescent dyes, roller, USB flash memory and QIAcuity Software Suite: includes 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts also included.	911001
QIAcuity One, 5plex Instrument	One-plate digital PCR instrument for detecting up to 5 fluorescent dyes, roller, USB flash memory and QIAcuity Software Suite: includes 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts also included.	911021
QIAcuity Four Instrument	Four-plate digital PCR instrument for detecting up to 5 fluorescent dyes,	911040

Product	Contents	Cat. no.
	notebook computer, barcode scanner, roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit.1 year warranty on labor, travel, and parts.	
QIAcuity Eight Instrument	Eight-plate digital PCR instrument for detecting up to 5 fluorescent dyes, notebook computer, barcode scanner, nanoplate roller, USB flash memory and QIAcuity Software Suite: includes installation, training, and 1 preventive maintenance visit. 1 year warranty on labor, travel, and parts.	911050
Related Products		
Nanoplate Seals (11)	11x nanoplate Seals	250099
PAXgene Blood ccfDNA Tubes	100 blood collection tubes (10 ml). To be used in conjunction with the QIAamp MinElute ccfDNA Kit, the QIAamp Circulating Nucleic Acid Kit (50) or the QIAsymphony PAXgene Blood ccfDNA Kit (192)	768115
Proteinase K (1 mL, 5 mL)	Proteinase K solution with a concentration of ≥20 mg/mL and an activity of ≥800 U/mL	RP107B-1 RP107B-5
QIAamp Circulating Nucleic Acid Kit (50)	The QIAamp Circulating Nucleic Acid Kit greatly simplifies concentration and purification of free-circulating DNA and RNA from plasma or serum. The kit can be automated on the QIAcube Connect.	55114

Product	Contents	Cat. no.
EZ1&2 ccfDNA Kit (48)	The EZ1&2 ccfDNA Kit provides easy and fully automated extraction of high-quality cfDNA from up to 8 ml of serum or plasma samples. Magnetic-bead technology allows parallel processing of 14 samples on the EZ1 Advanced XL or 24 samples on the EZ2 Connect.	954854
QIAsymphony DSP Circulating DNA Kit (192)	The QIAsymphony DSP Circulating DNA Kit provides reagent cartridges and reagents for fully automated and simultaneous purification of human circulating cell-free (ccf) DNA from human plasma and urine using the QIAsymphony SP instrument. Protocols are available for 2 and 4 ml sample volumes.	937556
QIAamp DNA Blood Mini Kit (50)	QIAamp DNA Blood Kits provide silica-membrane-based DNA purification from whole blood, plasma, serum and other body fluids. The kits are designed for a range of sample sizes from 200 µl up to 10 ml fresh or frozen human whole blood.	51104
EZ1&2 DNA Blood 200 µL Kit (48)	EZ1&2 DNA Blood Kits contain all required reagents and labware for rapid, automated purification of DNA from 200-350 µl whole blood samples or buffy coat samples using magnetic-particle technology. Reagents are supplied in prefilled and sealed reagent cartridges, which ensures speed and convenience in loading the instrument. EZ1&2 DNA Blood Kits are automated with the EZ2 Connect (1-24 samples per run). These kits can also	951034

Product	Contents	Cat. no.
	be used with the EZ1 Advanced XL (1–14 samples per run).	
QIAsymphony DSP DNA Midi Kit (96)	QIAsymphony DSP DNA Kits, in combination with the QIAsymphony SP, enable automated total DNA purification from human whole blood, buffy coat, tissues and FFPE tissues, as well as viral DNA from human whole blood for in vitro diagnostic use. Kits are available in mini and midi formats for sample volumes ranging from 200 µL to 1000 µL.	937255
QIAamp DNA FFPE Advanced Kit (50)	Increase your recovery of high-quality DNA from FFPE tissue with the QIAamp DNA FFPE Advanced Kits' xylene-free, no-wash deparaffinization, double-lyse protocol, and UCP (ultra-clean production) spin column technology.	56604
EZ1&2 DNA FFPE Kit (48)	Purify large amounts of amplifiable DNA from hard-to-lyse formalin-fixed, paraffin-embedded (FFPE) tissue. The EZ1&2 DNA FFPE protocol uses double lysis to recover DNA effectively, while the optional uracil-N-glycosylase (UNG) step removes deaminated cytosine artifacts to limit the risk of nucleotide read errors.	954404

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Document Revision History

Revision	Description
03/2024	Initial release.

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