Conclusion

Liquid biopsy samples can be challenging due to the low amounts of analytes such as ctDNA, the presence of ultra-rare variants and the need for high-sensitivity techniques for variant detection. In this Application Note, we demonstrated the utility of the QIAcuity Digital PCR System to confidently detect ultra-rare *PIK3CA* variants in cfDNA.

The manual QIAamp workflows and the automated EZ2 and QIAsymphony workflows delivered cfDNA with high yield and purity from large plasma volumes of up to 10 mL. The automated QIAGEN workflows also eliminated the need for manual pre-enrichment or preparation of plates.

Additionally, we demonstrated the comparability of dPCR and Qubit quantifications and the ability of dPCR to quantify *PIK3CA* mutation frequencies in cfDNA with high precision.

The cost and time-efficient analysis of cfDNA samples using QIAcuity dPCR make it an ideal tool for precise quantification of ultra-rare variants for various applications, including biomarker research, mutation screening, molecular characterization of blood samples and drug development.

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Application Note

Liquid biopsy-based detection of *PIK3CA* mutations from cfDNA using an end-to-end digital PCR workflow

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Introduction

PIK3CA is one of the most commonly mutated genes in human cancers and, therefore, a key target for cancer precision medicine. Identifying *PIK3CA* mutations from liquid or tissue biopsies can guide the use of therapies that target the aberrant signaling pathways activated by these mutations.

One of the major limitations of variant detection from cell-free DNA (cfDNA) is the ultra-low abundance (<1%) of circulating tumor DNA (ctDNA) within cfDNA (1), and traditional gold-standard methods like Pyrosequencing® or qPCR are often unable to deliver on such low limit of detection (LoD) requirements.

Digital PCR (dPCR) is a powerful technique that detects and quantifies ultra-rare mutations in a high background of wild-type cfDNA down to 0.1% variant allele frequency. Nanoplate-based dPCR systems, such as the QIAcuity® Digital PCR System, are particularly useful for ultra-rare variant detection as they allow larger eluate input volumes to achieve a lower LoD (2).

Here, we describe end-to-end manual and automated workflows that enable accurate detection and absolute quantification of ultra-rare *PIK3CA* variants in cfDNA using the QIAcuity Digital PCR System (Figure 1).

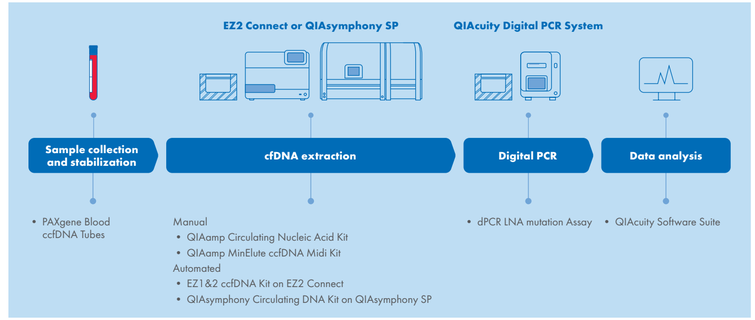

Figure 1. A fast and simple dPCR workflow for absolute quantification of mutations from cfDNA.

Sample to Insight

Materials and Methods

Sample collection and stabilization

Venous whole blood from consented, healthy donors was collected into PAXgene® Blood ccfDNA Tubes, and plasma was separated according to the manufacturer's instructions. The plasma pool was spiked with artificial cfDNA containing *PIK3CA* H1047R mutation to achieve a mutation frequency of ~0.5 % (5-Gene-Multiplex 1% AF cfDNA, *AKT1/BRAF/ERBB2/KRAS/PIK3CA*, sensID).

cfDNA extractions

Plasma samples were processed using manual and automated protocols using the respective maximum input volumes as specified below.

- **Manual using:**
  - QIAamp® Circulating Nucleic Acid Kit (5 mL)
  - QIAamp MinElute® ccfDNA Midi Kit (10 mL)
- **Automated using:**
  - EZ1&2™ ccfDNA Kit (10 mL) on the EZ2® Connect instrument
  - QIAsymphony® Circulating DNA Kit (10 mL) on the QIAsymphony SP instrument
  - Maxwell® RSC ccfDNA Plasma Kit (8 mL (Promega; eluted in PCR elution buffer) on the Maxwell RSC

Eluate volumes were set to <52 µL wherever possible or filled to 52 µL with water. An overview of QIAGEN® cfDNA extraction solutions is shown in Figure 2.

cfDNA yield quantification

cfDNA yields from each extraction method were determined using 2 µL of eluate and the Qubit™ 1x dsDNA HS Assay (Thermo Fisher Scientific). Additionally, absolute quantification of wild-type *PIK3CA* copies was carried out using the QIAcuity Digital PCR System.

dPCR and data analysis

For absolute quantification of mutant *PIK3CA* copies, eluates – 50 µL from each extraction method – were split into two wells of a 26k 24-well Nanoplate and analyzed for *PIK3CA* H1047R mutation using the dPCR LNA® Mutation Assay (DMH0000036) on the QIAcuity Digital PCR System. Results were analyzed using QIAcuity Software Suite (Suite 2.5.0.0).

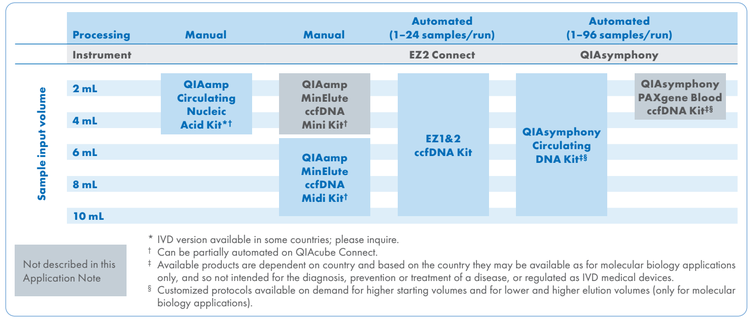

Figure 2. Choose the method that fits your sample input volume, throughput and automation needs. Our manual and automated cfDNA extraction protocols cover sample input volumes from 2–10 mL and throughputs from 1–96 samples per run.

Results and Discussion

Easy and reproducible extraction of high-yield cfDNA using manual and automated protocols

The manual QIAamp extraction methods and the automated protocols on EZ2 Connect and QIAsymphony SP yielded consistent and comparable cfDNA concentrations when measured using dPCR. The Qubit measurements, on the other hand, showed variations in cfDNA yields. For instance, the Qubit measurement of the QIAamp Circulating Nucleic Acid Kit is impacted by the use of carrier RNA during sample extraction and, therefore, unreliable. Moreover, cfDNA yields from the Maxwell RSC ccfDNA Plasma Kit were higher when quantified by dPCR than Qubit, which may indicate the presence of more ssDNA. Overall, the QIAamp Circulating Nucleic Acid Kit showed higher yields of cfDNA per mL plasma, in line with its recognition by the scientific community as the gold standard for cfDNA isolation (3–6) (Figure 3).

However, the total yield obtained using the QIAamp Circulating Nucleic Acid Kit was significantly lower (28 ng vs 42 ng) due to its lower input volume than the other QIAGEN kits. Thus, for manual isolations, we recommend the second-generation QIAamp MinElute ccfDNA Midi Kit, which allows the processing of up to 10 mL serum or plasma and additionally enables partial automation on the QIAcube® Connect instrument.

All QIAGEN methods allow low elution volumes for high concentrations of nucleic acids – a requirement for the successful detection of ultra-rare variants. In addition, the EZ1&2 ccfDNA and QIAsymphony Circulating DNA Kits enable fully automated processing of large sample volumes of up to 10 mL without manual pre-enrichment or preparation of plates or tubes. At the same time, the Maxwell protocol allows only up to 8 mL sample volumes and requires manual pre-enrichment and a centrifugation step.

Accurate detection of cfDNA variants using the QIAcuity dPCR System

The *PIK3CA* H1047R mutation frequency was calculated for all eluates by determining the ratio of detected mutation copies to mutated plus wild-type *PIK3CA* copies (Figure 4). A clear separation of mutated and wild-type copies can be seen in the 2D scatterplot obtained using the QIAcuity Software Suite (Figure 5).

Manual and automated sample preparation methods using QIAGEN kits enabled accurate and reliable variant detection on the QIAcuity dPCR System compared to the Maxwell protocol. The higher CI range for the QIAamp Circulating Nucleic Acid Kit indicates the lower plasma input, which decreases the precision and potential sensitivity of variant detection. ▷

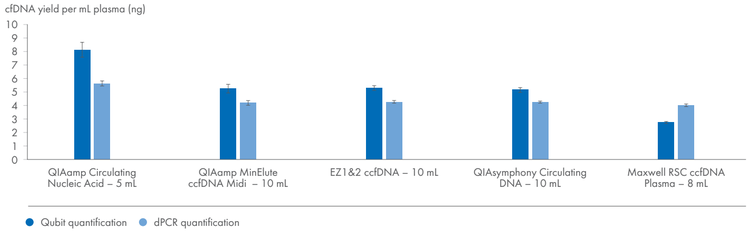

Figure 3. High yield of cfDNA. Yield per mL plasma quantified by Qubit dsDNA HS or dPCR for all extraction methods. The averages of two replicates are shown with standard deviations.

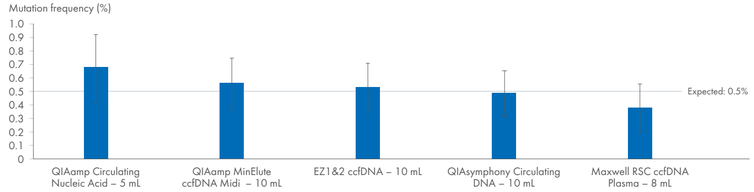

Figure 4. Accurate detection of mutation frequencies. *PIK3CA* H1047R mutation frequency was determined by a QIAcuity dPCR LNA Mutation Assay. The averages of two replicates are shown. Error bars indicate the 95% confidence interval (CI).

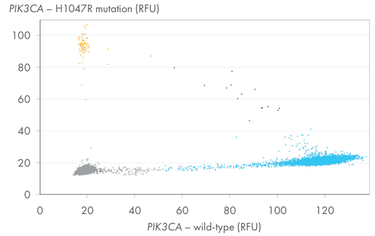

Figure 5. Mutation analysis of cfDNA using the QIAcuity Digital PCR System. Differences can be visualized at high resolution in the 2D scatterplot obtained using the QIAcuity Software Suite. The fluorescence intensity (RFU) of wild-type *PIK3CA* (x-axis) was plotted against the fluorescence intensity of *PIK3CA* – H1047R mutation (y-axis).

Digital PCR is a powerful technology that enables precise detection of mutation frequencies down to 0.1% in a wild-type background. dPCR systems like the QIAcuity that allow high template addition volumes, and thus higher template analyzed volumes, can provide the high sensitivity and accuracy needed for ultra-rare variant detection. Additionally, the QIAcuity offers multiplexing capabilities that allow the detection of multiple targets in up to 5 channels. The QIAcuity eight-plate dPCR instrument can also accommodate up to 8 nanoplates. There are various dPCR assays available for CNV detection, mutation or biomarker analysis, as well as established assay designs for reference genes and housekeeping genes. All dPCR products are compatible with QIAGEN downstream and upstream technologies.