Fully automated cell-free DNA extraction from up to 8 mL plasma enables sensitive mutation detection with digital PCR and NGS

Simon Hertlein[1], Annika Voets[1], Julius Albers[1], Oezlem Karalay[1], Zhong Wu[2], Martin Schlumpberger[1], Markus Sprenger-Haussels[1]

[1] QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, Germany

[2] QIAGEN Sciences LLC, 6951 Executive Way, Frederick, MD 21703, USA

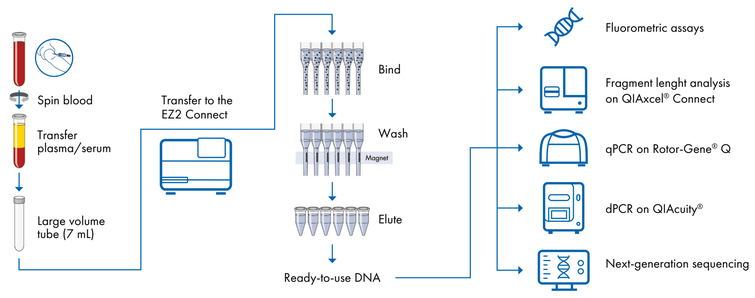
Abstract

Cell-free DNA (cfDNA) is found in blood, urine and other body fluids and is commonly characterized as extracellular DNA fragments shorter than 1000 bp. cfDNA is an important analyte for screening and treatment monitoring, for example in cancer research. Since cfDNA is present in very low amounts, large input volumes are required, and a high degree of automation is needed to increase sample throughput. The new EZ1&2 ccfDNA process uses magnetic beads to enable efficient purification of cfDNA from 0.5–8 mL human plasma in 39–73 minutes. No manual pretreatment or pre-enrichment is needed and up to 24 samples can be processed in parallel, resulting in eluates ready to use for downstream applications such as qPCR, dPCR, fragment length analysis and NGS.

In this study, cfDNA from plasma of healthy donors was extracted using the EZ1&2™ ccfDNA Kit. cfDNA yield and quality were determined using fluorometric assays, qPCR, dPCR and fragment length analysis, in comparison to other cfDNAextraction solutions. cfDNA material with defined mutations was spiked into healthy donor plasma and eluates purified using the EZ1&2 ccfDNA Kit were analyzed for detection of variants with allele frequencies (VAF) below 0.5 % using dPCR. Furthermore, using SeraCare Reference material, the new QIAseq® Targeted cfDNA Ultra Panel was evaluated for low VAF detection using eluates from the EZ1&2 ccfDNA Kit.

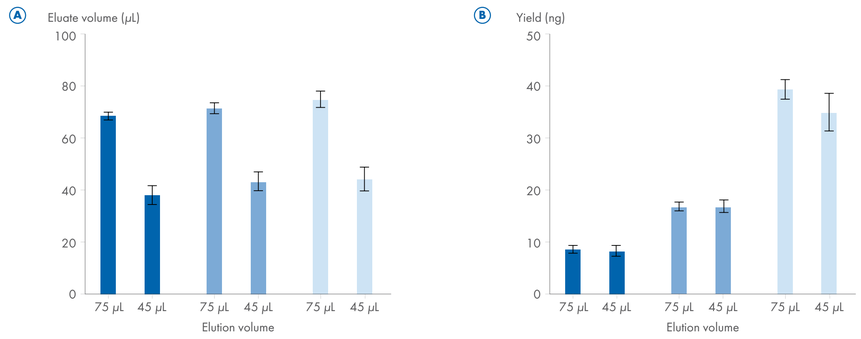
Here, we show that cfDNA can be efficiently isolated from 0.5–8 mL plasma using the EZ1&2 ccfDNA Kit with either 75 µL or 45 µL elution volumes, with no discernible inhibitory effects in fluorometric assays, qPCR, dPCR, fragment length analysis or NGS-based methods. This fully automated cfDNA extraction method proved to be equal to or better than other cfDNA extraction methods and enabled the detection of VAFs as low as 0.1% in dPCR and NGS.

Comprehensive and integrated workflow



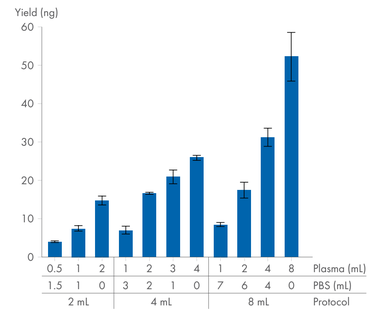
Workflow from blood collection to cfDNA analysis using the EZ1&2 ccfDNA Kit. After generation of plasma, up to 4 mL are transferred into one Large Volume Tube (LVT; two LVTs with 4 mL each are used for the 8 mL protocol) and cfDNA is automatically extracted on the EZ2® Connect instrument, ready-to-use for downstream analyses including fluorometric quantification, fragment length analysis, qPCR, dPCR and NGS applications.

Consistent elution volumes and yields

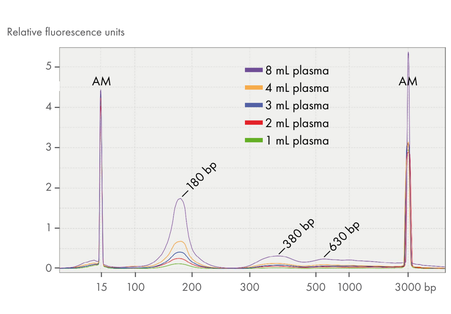


Choice of elution volumes and consistent yields. Pooled EDTA-plasma was extracted using either the 2 mL, 4 mL or 8 mL protocol with a 75 µL or 45 µL elution volume on the EZ2 Connect. **A** The measured eluate volumes were consistent within the range of the selected elution volume and **B** both elution volumes resulted in comparable yields. Yields were quantified using the Investigator® Quantiplex® Pro RGQ Kit (91 bp human fragment). Average ± standard deviation is shown, n = 4.

Flexible starting volumes and linearity of yields

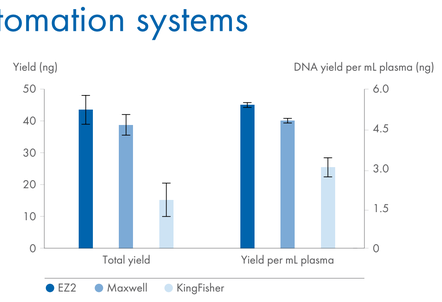


Linear input. Different amounts of plasma were adjusted with PBS and cfDNA was extracted using the 2 mL, 4 mL or 8 mL protocol on the EZ2 Connect demonstrating the flexibility of starting volumes and a linear increase in yields. Yields were quantified using the Investigator Quantiplex Pro RGQ Kit (91 bp human fragment). Average ± standard deviation is shown; n = 3.

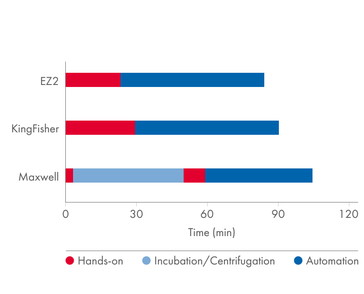


Fragment length analysis on QIAxcel Connect. cfDNA eluates from the first experiment were analyzed on the QIAxcel Connect using the DNA High-Sensitivity Cartridge. The mononucleosomal peak (180 bp) increases with plasma input and the di- (380 bp) and trinucleosomal (630 bp) peaks can be observed at larger input volumes. AM: Alignment marker.

High cfDNA extraction performance compared with other automation systems



Performance vs. automated systems from other suppliers. 8 mL (EZ2 and Maxwell®) or 5 mL (KingFisher™) plasma from PAXgene® Blood ccfDNA Tubes was used for cfDNA extraction using different extraction methods. cfDNA yield (total yield on the left or yield per mL plasma on the right) was quantified by the Investigator Quantiplex Pro RGQ Kit (91 bp human fragment). Average ± standard deviation is shown; n = 3.

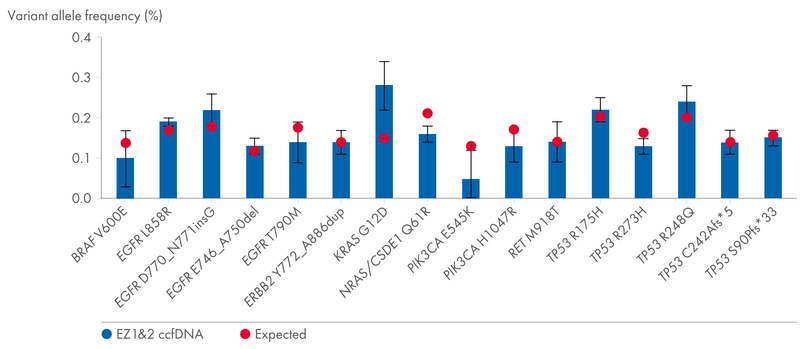


Workflow comparisons for maximum plasma input. Comparison of the different workflow times for processing of 12 samples with maximum possible plasma input (EZ2 and Maxwell 8 mL, KingFisher 5 mL). The EZ1&2 ccfDNA procedure is fully automated, whereas the Maxwell RSC ccfDNA LV Plasma Kit requires manual pre-enrichment steps and the MagMAX™ Cell-Free DNA Isolation Kit needs manual pre-dispensation of reagents into plates.

High accuracy and sensitivity for dPCR-based mutation detection with EZ2 Connect and QIAcuity

High accuracy and sensitivity in dPCR. Plasma from PAXgene Blood ccfDNA Tubes (8 mL for EZ2 Connect, 5 mL for KingFisher) was spiked with increasing amounts of mutation copies (sensID) and cfDNA was extracted using the EZ2 Connect or the KingFisher instrument. The number of mutation copies in total eluates was determined by dPCR on the QIAcuity or ddPCR on the Bio-Rad QX200 using the *PIK3CA* p.H1047R assay. The combination of EZ2 Connect cfDNA extraction and QIAcuity dPCR offers higher accuracy and sensitivity for mutation detection than kits and instrumentation from other suppliers.

Accurate, sensitive detection of low-frequency variants using the QIAseq Targeted cfDNA Ultra Panel



Sensitive detection of low-frequency variants. Seraseq® ctDNA Reference Material v2 AF0.125% was extracted using the 8 mL protocol on the EZ2 Connect. Approximately 50 ng cfDNA was used as input for QIAseq Targeted cfDNA Ultra Lung Cancer Panel library preparation, and libraries were sequenced using a NextSeq® 500/550 Mid Output v2.5 Kit (300 Cycles; Illumina, Inc.). The detected variant allele frequencies (VAFs) here represent average ± standard deviation of n = 3 compared with the expected VAF in the material given by SeraCare (red dots).

Conclusions

- EZ1&2 ccfDNA workflow enables fully automated cfDNA extraction from large plasma volumes up to 8 mL
- Large Volume Tubes and prefilled cartridges eliminate manual pre-enrichment or preparation of plates
- Flexible starting volumes in the same run and different elution volumes with consistent yields
- High performance with shorter run times vs. suppliers with semi-automated solutions
- Integration with the QIAcuity dPCR platform optimizes the detection and quantification of rare mutations for highest sensitivity and reproducibility
- Mutation detection down to 0.1 % variant allele frequency using QIAseq Targeted cfDNA Ultra Panel next-generation sequencing

The EZ1&2 ccfDNA Kit is intended for molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit instructions for use or user operator manual. QIAGEN instructions for use and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services (or your local distributor).

Experiments were conducted at QIAGEN GmbH, QIAGEN Strasse 1, 40724 Hilden, Gemany.

Sample to Insight