Technical Information

# QIAsymphony® DSP Circulating DNA Kit – Detection of low-abundance cfDNA using Droplet Digital® PCR

## Introduction

The QIAsymphony® DSP Circulating DNA system constitutes a ready-to-use in vitro system for the qualitative purification of human (circulating) cell-free DNA (cfDNA) from human plasma and urine. Cell-free DNA is a key analyte for liquid biopsy samples. Due to very low concentrations of cfDNA found in plasma, representing a limited number of molecules available for use as biomarkers in a sample, high sample input volumes and reliable quantification methods are required to maximize sensitivity. Here, we evaluate the performance of the QIAsymphony DSP Circulating DNA Kit (cat. no. 937556) with Droplet Digital® PCR (ddPCR®) as the downstream application in 2 studies and a customer submission.

## Methods overview

Cell-free DNA was extracted on the QIAsymphony SP instrument (cat. no. 9001297) using various plasma input volumes up to 10 ml. Plasma was spiked with a dilution series of controls to estimate extraction efficiency of low-abundance biomarkers. Quality and quantity of cfDNA was analyzed by the Qubit® dsDNA HS Assay Kit, qPCR and ddPCR.

For quantification of total cell-free DNA using qPCR and ddPCR, primers and probes specific for human 18S rRNA coding region were used. In addition, for quantification of male-specific cell-free DNA, primers and probes specific for the Y-chromosome–specific multi-copy gene *DYS14* and single-copy gene *SRY1* were used. Primer and probe sequences are provided in Table 1, next page. Table 1 also includes primer and probe information for the mutation detection (*AKT1* E17K) and copy number detection (*HER2* [1]) assays.

The qPCR assay was performed on a Rotor-Gene® Q instrument using the QuantiTect® Multiplex PCR NoRox Kit (QIAGEN, cat. no. 204741) according to the manufacturer's instructions. The ddPCR assay was performed on a Bio-Rad® QX200™ AutoDG™ Droplet Digital PCR System using the ddPCR Supermix for Probes (No dUTP) (Bio-Rad, cat. nos. 1863023 and 1863024) and the ddPCR Supermix for Probes (Bio-Rad, cat. no. 1863010) according to the manufacturer's instructions.

Detailed methods are provided in the study-specific sections of this document.

**Table 1. Primers and probes used in qPCR and ddPCR assays**

| Primer/probe name | Sequence | Probe labels |
|---|---|---|
| 18S 66 bp forward | GCCGCTAGAGGTGAAATTCTTG | – |
| 18S 66 bp reverse | CATTCTTGGCAAATGCTTTCG | – |
| 18S 66 bp probe | ACCGGCGCAAGACGGACCAGA | qPCR: 5' Bodipy – BHQ2 3'<br>ddPCR: 5' HEX – BHQ1 3' |
| *SRY1* 78 bp forward | TCCTCAAAAGAAACCGTGCAT | – |
| *SRY1* 78 bp reverse | AGATTAATGGTTGCTAAGGACTGGAT | – |
| *SRY1* 78 bp probe | CACCAGCAGTAACTCCCCACAACCTCTTT | ddPCR: 5' FAM – BHQ1 3' |
| *DYS14* 66 bp forward | GAGCAGGCGTGGGTACTATTG | – |
| *DYS14* 66 bp reverse | GTCTGCTGCTCGGCATCAC | – |
| *DYS14* 66 bp probe | CCTGCATGCGGCAGAGAAACCC | ddPCR: 5' FAM – BHQ1 3' |
| *HER2* forward (1) | ACAACCAAGTGAGGCAGGTC | |
| *HER2* reverse (1) | GTATTGTTCAGCGGGTCTCC | |
| *HER2* probe (1) | CCCAGCTCTTTGAGGACAAC | ddPCR: 5' FAM – MGB NFQ 3' |
| *EFTUD2* forward (1) | GGTCTTGCCAGACACCAAAG | |
| *EFTUD2* reverse (1) | TGAGAGGACACACGCAAAAC | |
| *EFTUD2* probe (1) | GGACATCCTTTGGCTTTTGA | ddPCR: 5' VIC – MGB NFQ 3' |
| *AKT1* WT for p.E17K | dHsaCP2000032 PrimePCR™ ddPCR Mutation Assay, Human (Bio-Rad) | |
| *AKT1* p.E17K | dHsaCP2000031 PrimePCR ddPCR Mutation Assay, Human (Bio-Rad) | |

## Results overview

Linearity in cfDNA recovery, as well as robust detection of low-abundance biomarkers, was observed for different sample input volumes ranging from 1–10 ml. Cell-free DNA yield and sensitivity were also comparable to results from an in-house QIAGEN manual cfDNA extraction kit. Customer-submitted data shows the QIAsymphony DSP Circulating DNA Kit combined with subsequent ddPCR has been successful for detecting somatic mutations in circulating tumor DNA in a clinical trial setting.

Details of specific results are provided in the respective study sections of this document.

## Detection of male cfDNA in spiked female plasma using Droplet Digital PCR

Basic performance of the QIAsymphony DSP Circulating DNA Kit was evaluated with the focus on extraction of low-abundance cfDNA in a background of normal cfDNA levels.

### Methods

Whole blood was collected from healthy donors in 10 ml BD® Vacutainer® K2EDTA tubes. Female and male plasma were generated according to CEN-TS 16835-3. Female plasma was spiked

with male plasma (0–1024 µl range) to a total volume of 2 ml as illustrated in Figures 1 and 2, below and next page. Afterwards, cfDNA was extracted using the QIAsymphony DSP Circulating DNA Kit according to the manufacturer's instructions, unless stated otherwise. The cfDNA yield was determined with a ddPCR assay for male-specific genes located on the Y-chromosome using the Bio-Rad QX200 AutoDG Droplet Digital PCR System. A dilution series was used to determine the Limit of Detection (LoD) for the multi-copy gene *DYS14*. The same eluates were used to determine the LoD for the single-copy gene *SRY1* in parallel.

## Results

Negative plasma samples (female plasma) were used to determine the LoD for the Y-chromosome–specific genes *DYS14* and *SRY1* (red line in Figures 1 and 2). The calculated LoD for *DYS14* of 7.3 cps/well (= 5.7 *DYS14*-positive droplets) equates to 2 µl male plasma spiked into female plasma to a total volume of 2 ml (Figure 1). Moreover, a high accuracy for the linear increase in cps/well for *DYS14* ($R^2$ = 0.9974), which indicates a reliable extraction of low-abundance genes, was calculated for the spiked plasma above the LoD (2–1024 µl range). Based on the number of droplets, the target copy number per well is calculated as shown in Figure 1. The fluorescence signals for *DYS14*, as detected by ddPCR, for the individual spiked plasma samples are shown in Figure 3, next page.

Due to the lower number of DNA copies, 16 µl of male plasma is required for specific detection of the single-copy gene *SRY1* in a total volume of 2 ml female plasma (calculated LoD 3.2 cps/well) (Figure 2). Again, a high accuracy above the LoD for the linear increase in cps/well ($R^2$ = 0.9864) was calculated.

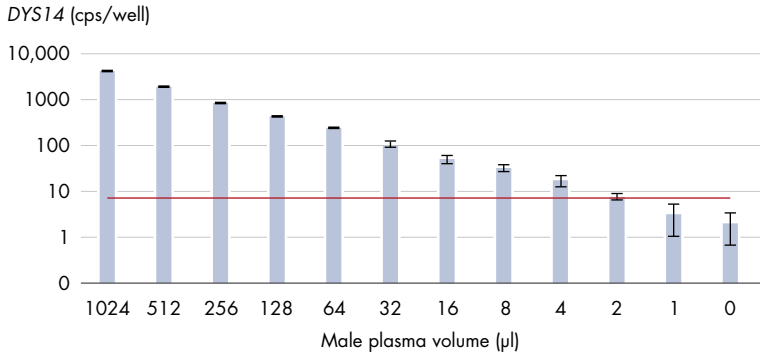



**Figure 1. Determination of LoD for *DYS14* using Droplet Digital PCR.** Female plasma was spiked with male plasma ranging from 0 to1024 µl, up to a total volume of 2 ml. Cell-free DNA was extracted using the QIAsymphony DSP Circulating DNA Kit. Spiked female plasma was extracted in 4 replicates. Negative plasma (non-spiked female plasma) was extracted in 10 replicates to determine LoD. Male circulating DNA yield was determined by ddPCR (Y-chromosome–specific gene *DYS14* [multi-copy gene]) with the Bio-Rad QX200 AutoDG Droplet Digital PCR System using a fixed volume of 8 µl DNA. Results were calculated as target copies per well. Calculation of LoD for *DYS14* is based on non-spiked female plasma (LoD = mean + 4x SD; red line indicates calculated LoD of 7.3 cps/well).

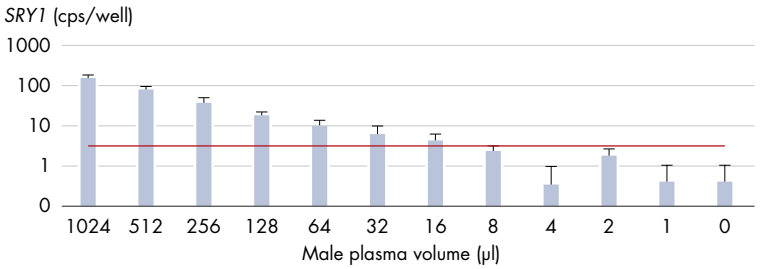
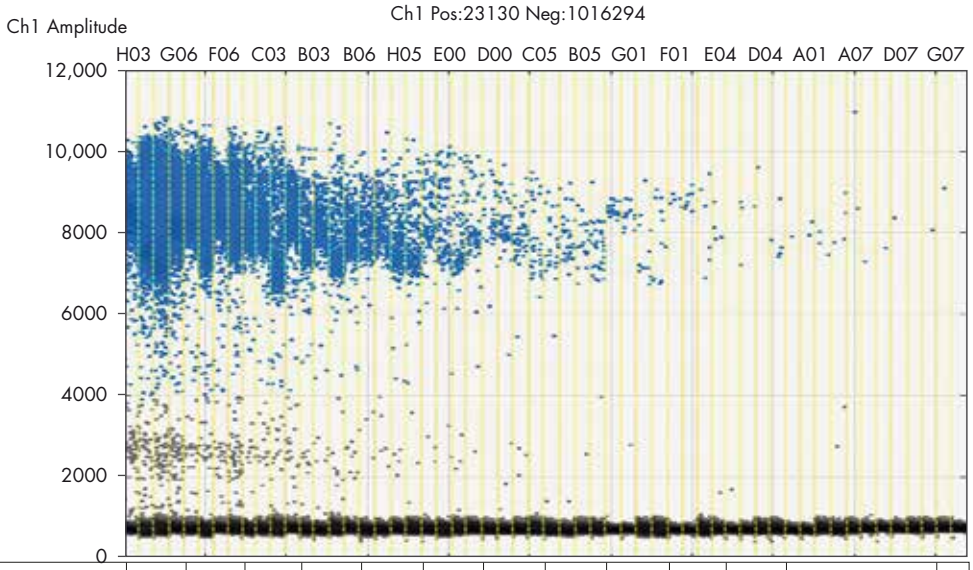



**Figure 2. Determination of LoD for *SRY1* using Droplet Digital PCR.** Female plasma was spiked with male plasma ranging from 0 to 1024 µl, up to a total volume of 2 ml. Cell-free DNA was extracted using the QIAsymphony DSP Circulating DNA Kit. Female plasma was extracted in 4 replicates. Male circulating DNA yield was determined by ddPCR (Y-chromosome–specific gene *SRY1* [single-copy gene]) with the Bio-Rad QX200 AutoDG Droplet Digital PCR System using a fixed volume of 8 µl DNA. Results were calculated as target copies per well. Calculation of LoD for *SRY1* is based on non-spiked female plasma (LoD = mean + 4x SD; red line indicates calculated LoD of 3.2 cps/well).




| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Positive droplets** | 3056 | 1370 | 637 | 341 | 200 | 85 | 40 | 27 | 14 | 6 | 3 | 1.6 | 0.5 |
| **Female plasma (µl)** | 976 | 1488 | 1744 | 1872 | 1936 | 1968 | 1984 | 1992 | 1996 | 1998 | 1999 | 2000 | NTC |
| **Male plasma (µl)** | 1024 | 512 | 256 | 128 | 64 | 32 | 16 | 8 | 4 | 2 | 1 | 0 | NTC |
| **Replicates** | 4 | | | | | | | | | | | 10 | 2 |

**Figure 3. Number of positive droplets for *DYS14* for female plasma spiked with male plasma.** Female plasma was spiked and processed, and circulating DNA yield was determined as described in Figure 1. Graphic shows number and fluorescence amplitude for negative droplets (black) and *DYS14*-positive droplets (blue) measured with ddPCR.

## Linearity and reliable detection of low-abundance cfDNA using Droplet Digital PCR

The experimental design (analysis of Y-chromosome–specific genes at the limit of detection in a normal cfDNA background) described in the previous section, "Detection of male cfDNA in spiked female plasma using Droplet Digital PCR", was used as a model for this study to demonstrate reliable detection of low-abundance genes depending on sample input volume.

## Methods

Female plasma was spiked with male plasma followed by extraction of cfDNA from different plasma volumes ranging from 1–10 ml. To ensure more reliable detection of *DYS14*, female plasma was spiked with 2 µl male plasma per ml of female plasma (equates to 2x LoD of *DYS14*) to minimize risk of dropouts (i.e., no *DYS14*-positive droplets) for the low plasma input volumes. For a better assessment of the extraction efficiency using the QIAsymphony DSP Circulating DNA Kit, cfDNA from 4 ml plasma input was also extracted with an in-house cfDNA extraction kit, serving as a manual reference method (indicated as red plots in Figures 4 and 5, below and next page). First, total cfDNA yield was determined using two independent methods: Qubit dsDNA HS Assay Kit (Figure 4A) and an in-house validated real-time PCR system, which amplifies a 66 bp amplicon from the 18S rDNA (Figure 4B). Secondly, reliable detection of the multi-copy gene *DYS14* in the female background with ddPCR was analyzed (Figure 5A) as described in the previous section ("Detection of male cfDNA in spiked female plasma using Droplet Digital PCR"). In addition, the single-copy gene *SRY1* was analyzed (Figure 5B).

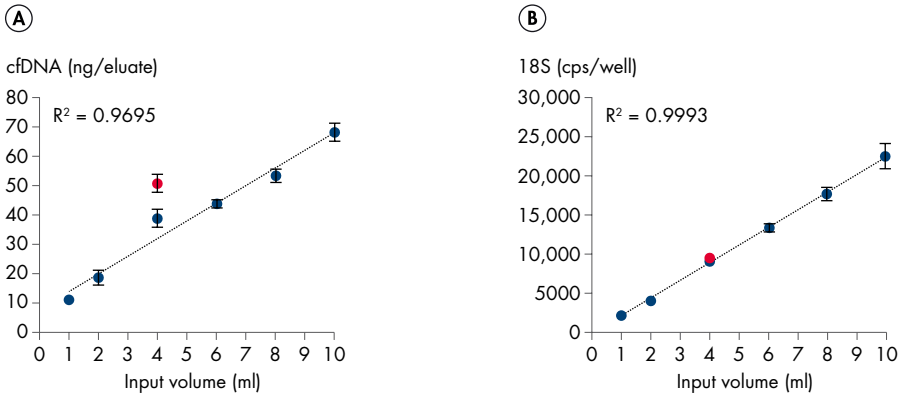



**Figure 4. Linearity in cfDNA recovery for sample input volumes from 1–10 ml.** Female plasma was spiked with male plasma at 2x LoD of *DYS14* (described in Figure 1). Cell-free DNA was extracted from 1–10 ml spiked female plasma (2 µl male plasma per ml female plasma) in 4 replicates using the QIAsymphony DSP Circulating DNA Kit (blue plots). Additionally, cfDNA was extracted from 4 ml spiked plasma in 8 replicates using an in-house cfDNA extraction kit serving as a manual reference method (red plots). Total circulating DNA yield was determined by **A** Qubit dsDNA HS Assay Kit and **B** real-time PCR (18S rDNA, multi-copy gene).

## Results

The linear increase in *DYS14* copies with increased sample input volume is shown in Figure 5A. As previously described, approximately 16 µl male plasma in a total volume of 2 ml female plasma (i.e., 8 µl/ml plasma) are required for determination of *SRY1* at the lower limit of detection. Due to the lower input of 2 µl per ml plasma, *SRY1* was analyzed below the calculated LoD. Therefore, only a hit rate study (presence of droplets) was executed for the different input volumes and results are summarized in Figure 5B.

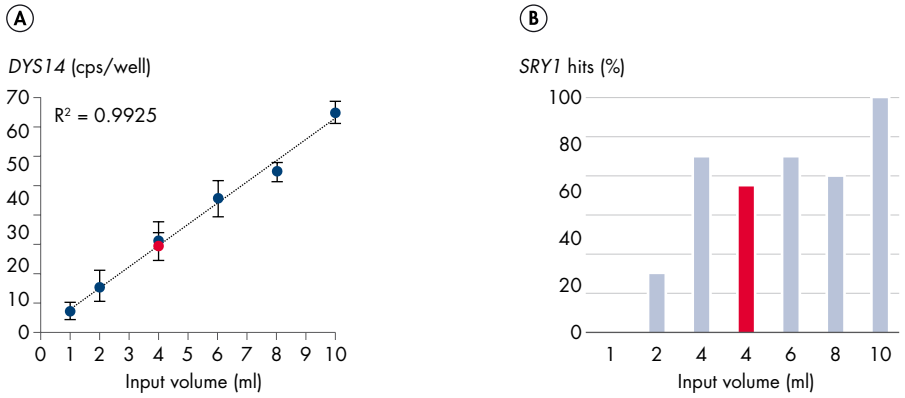



**Figure 5. Linearity in yield for low-abundance cfDNA depending on plasma input volume.** Female plasma was spiked with male plasma at 2x LoD of *DYS14* (described in Figure 1). Cell-free DNA was extracted from 1–10 ml spiked female plasma (2 µl male plasma per ml female plasma) in 4 replicates using the QIAsymphony DSP Circulating DNA Kit (blue plots and bars). Additionally, cfDNA was extracted from 4 ml spiked plasma in 8 replicates using an in-house cfDNA extraction kit serving as manual reference method (red plot and bar) (described in Figure 4). Circulating DNA yield was determined by ddPCR with the Bio-Rad QX200 AutoDG Droplet Digital PCR System using a fixed volume of 8 µl DNA. **A** Results were calculated as target copies per well for the Y-chromosome–specific gene *DYS14* (multi-copy gene). **B** Results were calculated in percentage of hits (positive droplets) for the Y-chromosome–specific gene *SRY1* (single-copy gene).

### Discussion

Figure 4 illustrates a linear increase in total cfDNA yield, detected with two independent methods, for extraction of 1–10 ml sample volumes using QIAsymphony SP and the QIAsymphony DSP Circulating DNA Kit. The validated in-house qPCR revealed more reliable results for the linearity, as well as correlation to the manual cfDNA extraction kit, compared to the Qubit dsDNA HS Assay Kit.

Figure 5A reveals reliable extraction at the lower limit of detection (1 ml and 2 ml plasma volumes) for the low-abundance gene *DYS14* and, in addition, a linear increase in yield for the elevated input volumes up to 10 ml plasma. A comparable extraction efficiency was obtained for the reference (the in-house cfDNA extraction kit) for 4 ml plasma input.

Figure 5B highlights the impact of an increased sample input volume on the detectability of the second low-abundance gene *SRY1*. Using lower plasma input volumes resulted in unreliable detection of positive droplets in ddPCR, whereas sensitivity of the assay can be increased by using the highest input volume (10 ml plasma) for cfDNA extraction on QIAsymphony SP.

## Customer-submitted data from routine testing

Using the QIAsymphony DSP Circulating DNA Kit with subsequent ddPCR as the downstream application was also tested by customers* in clinical trial settings. Cell-free DNA was extracted from plasma generated from EDTA blood collection tubes, as well as Streck® Cell-Free DNA BCT® Tubes (www.streck.com). Depending on the required sensitivity in the ddPCR downstream assay, cfDNA was extracted from 4 ml or 8 ml plasma volumes. Depending on the assay, mutations can

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be detected at allele frequencies as low as 0.08%. Overall, the QIAsymphony DSP Circulating DNA Kit provides reproducible results, both for DNA concentration in duplicate samples and also for downstream mutation and copy number detection.

In the following sections, "Mutation detection in cfDNA using Droplet Digital PCR" and "Copy number detection in cfDNA using Droplet Digital PCR", we provide typical examples of the resulting 2D plots that highlight the fluorescence signals for the probes (Figures 6 and 7, below and next page). For detailed information on primers and probes, refer to Table 1 (page 2).

## Mutation detection in cfDNA using Droplet Digital PCR

Figure 6 shows the *AKT1* E17K mutation detected in cfDNA extracted from plasma. The fluorescence signal on the Y-axis indicates the blue mutant droplets and the fluorescence signal on the X-axis represents the green wild-type droplets. In this instance, the mutation is present at 1.5% (i.e., 10 mutant droplets, 666 WT droplets).

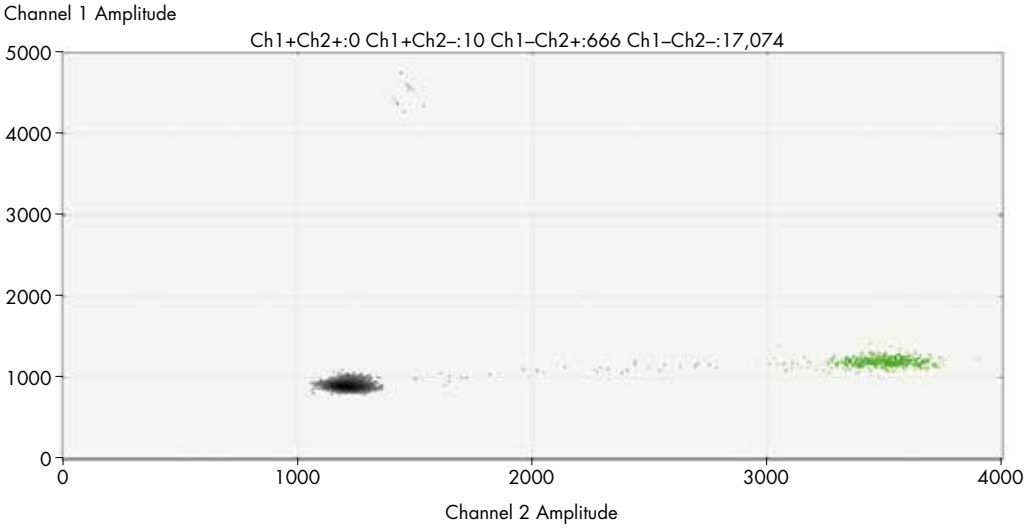


**Figure 6. Detection of somatic mutations in cfDNA from clinical samples.** Cell-free DNA was extracted from an 8 ml clinical plasma sample using the QIAsymphony DSP Circulating DNA Kit and eluted in 120 µl volume. Concentration of cfDNA was determined using the Qubit dsDNA HS Assay Kit (0.469 ng/µl). Detection and mutation frequency of *AKT1* was determined by ddPCR with the Bio-Rad QX200 AutoDG Droplet Digital PCR System using a fixed volume of 7.5 µl DNA. The 7.5 µl DNA input represents a 0.5 ml equivalent of plasma. Results are shown as a 2D plot: *AKT1* E17K-positive droplets (Y-axis, blue, 10 counts), *AKT1* WT-droplets (X-axis, green, 666 counts) and empty droplets (black, 17,074 counts).

## Copy number detection in cfDNA using Droplet Digital PCR

Figure 7 shows *HER2* amplification detected in cfDNA extracted from plasma. The fluorescence signal on the Y-axis indicates the blue droplets for *HER2* and the florescence signal on the X-axis represents the green reference gene droplets for *EFTUD2*. Here, *HER2* is amplified with a ratio of 4.75 (i.e., 3155 *HER2* droplets, 727 reference gene droplets). ▷

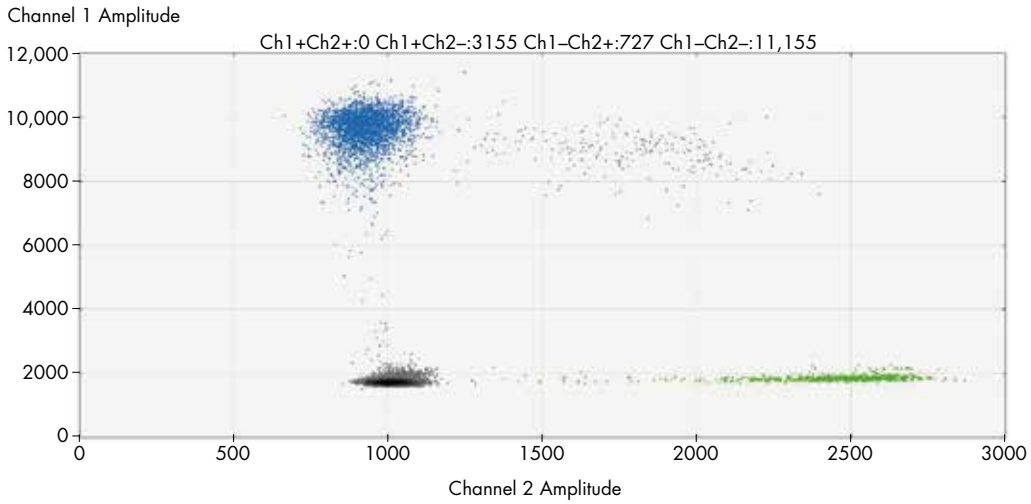


**Figure 7. Detection of copy number changes in cfDNA from clinical samples.** Cell-free DNA was extracted from an 8 ml clinical plasma sample using the QIAsymphony DSP Circulating DNA Kit and eluted in 120 µl volume. Concentration of cfDNA was determined using the Qubit dsDNA HS Assay Kit (5.08 ng/µl). Detection of *HER2* was determined by ddPCR using the Bio-Rad QX200 AutoDG Droplet Digital PCR System. Maximum cfDNA input for copy number assays is 10 ng (in this instance, 2 µl DNA was used in the reaction). Results are shown as a 2D plot: *HER2* droplets (Y-axis, blue, 3155 counts), *EFTUD2* reference gene droplets (X-axis, green, 727 counts) and empty droplets (black, 11,155 counts).

## Conclusion

The QIAsymphony DSP Circulating DNA Kit demonstrates reliable cfDNA extraction and a high recovery efficiency that are suitable for robust detection of low-abundance biomarkers using Droplet Digital PCR.

**References**

1. Garcia-Murillas, I., Lambros, M., and Turner, N.C. (2013) Determination of *HER2* amplification status on tumour DNA by digital PCR. PLoS ONE 8(12), e83409. doi:10.1371/journal.pone.0083409.

The QIAsymphony DSP Circulating DNA Kit is intended for in vitro diagnostic use.

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