Robust qPCR assay for validating effective isolation of cell-free DNA

Jolinde van Strien, Bram Luiken, Bo Rood, Lara van der Klugt, Eleni Draina, Pascal van der Weele
GenDx, Utrecht, The Netherlands

Introduction

Determining the donor-derived fraction of cell-free DNA (dd-cfDNA) in whole blood enables a less invasive monitoring regime for graft damage in organ transplant recipients. To prevent skewing of the perceived donor cfDNA fraction by genomic DNA (gDNA) contamination upon cfDNA isolation, a cfDNA quality control is required. Here, we present a quality control prototype assay (gFreeQC) that determines cfDNA purity while quantifying isolation yield through duplex detection of two targets: a long fragment to indicate genomic DNA contamination and a short fragment to represent total DNA (Figure 1). gFreeQC is fast, requires low DNA input and can be adapted to balance input volume and required sensitivity (Table 1).

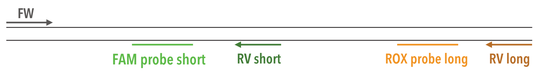

Figure 1. Assay principle

Method

For development purposes, gDNA was sheared using sonication to mimic the properties and sizing of cfDNA. This material was subsequently mixed with intact gDNA at various ratios to simulate gDNA contamination. A 50 cycle qPCR was performed using ROX detection for the long fragment and FAM detection for the short fragment. All reactions were performed in duplicate wells, each containing 2 µL of DNA input. Quantification was achieved by interpolation on a calibration curve created with a dilution series of 20 pg/µL – 20 ng/µL genomic DNA included in the same run.

Samples per run	Up to 42
Turn-around time	~1.5 hours
Hands-on time	10-20 minutes (could be automated)
Device	QuantStudio 6 or 7 Flex
Default input	4 µL (2 µL per well)
Flexible input	2 – 15 µL per well
Measuring interval	20 pg/µL – 20 ng/uL
Sensitivity	Input-dependent (Table 2)

Table 1. Assay specifications

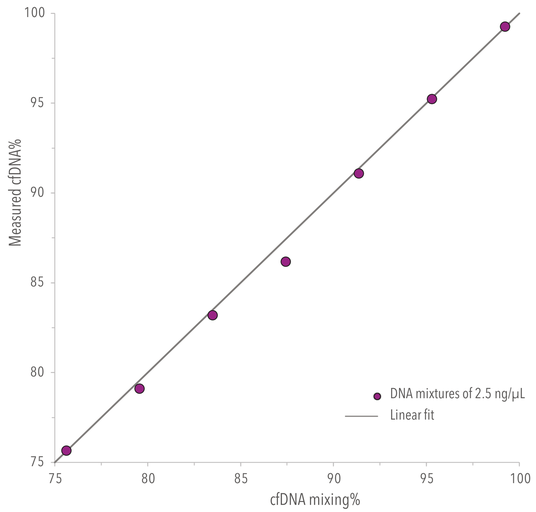

Figure 2. Linearity of cfDNA%. Sheared DNA is mixed with gDNA in different ratios. Measured cfDNA% was compared to the cfDNA% predicted based on the mixing ratio and the linear fit demonstrates the linear dependence.

DNA input (ng/µL)	Upper limit cfDNA%		Precision (CV%)	
	2 µL/well	5 µL/well	Within-run	Between-run
0.1	80%	92%	7.5%	8.3%
0.5	96%	98%	3.9%	5.1%
2.5	99.2%	99.7%	1.6%	1.6%

Table 2. Input-dependent sensitivity and precision. The upper detection limit for quantifiable cfDNA% increases with increasing DNA input concentration and volume. Precision CVs were determined based on four replicate measurements of 80% pure cfDNA samples: on the same calibration curve for within-run precision and on independent calibration curves for between-run precision.

Results

The gFreeQC assay can measure samples containing 0.1 to 20 ng/µL of DNA with cfDNA purity levels of up to 99.7%, depending on the input (Table 2). The assay shows an absolute deviation from linearity of 0.9%. (Figure 2). Furthermore, high precision was demonstrated, with CVs of 1.6%-8.3% depending on the input amount (Table 2). The observed cfDNA purity % was comparable when either 20% less or 20% more enzyme was used in the assay as compared to the standard enzyme input (Figure 3), indicating that even at the lowest input level, the assay performs robustly regardless of expected levels of variation in PCR efficiency.

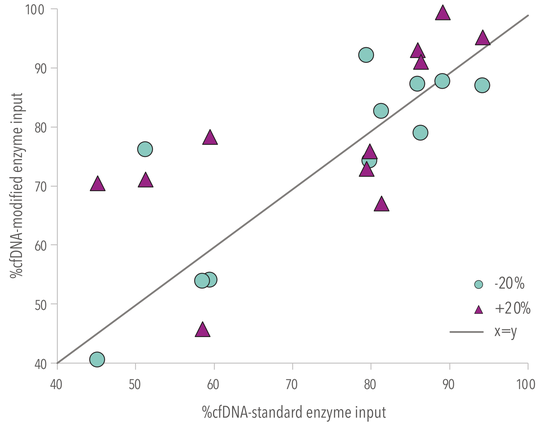

Figure 3. Assay robustness. Samples containing 0.1 ng/µL DNA with varying cfDNA concentrations were measured using the standard enzyme concentration as well as with 20% less and 20% more enzyme, simulating variation in PCR efficiency. The results of both non-standard conditions were compared to the results of the standard condition.

Conclusion

gFreeQC can determine cfDNA purity in low-concentration samples with high precision in a linear fashion. The assay could be used in the context of multiple cfDNA applications, including dd-cfDNA monitoring assays.

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