A New Universal Multiplex Digital PCR Method with Improved Precision for the Quantification of Donor Derived Graft cfDNA Traces

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Abstract

Background:

Graft-derived cell-free DNA (GcfDNA) quantification is gaining high interest as biomarker for graft integrity after transplantation. Using high throughput sequencing (HTS) (1) or digital PCR (dPCR) (2), the quantification of informative SNPs in total cell-free plasma DNA (cfDNA) became feasible. Molecule counting as employed by both methods enables the detection of small changes in GcfDNA levels with higher precision than qPCR. However, both methods include a preamplification using adapters ligated to the DNA, which introduces non-systematic amplification variability. Here we present a preamplification-free, multiplexed dual dPCR assay approach yielding a low overall imprecision.

Methods:

Informative SNP assays were selected for each patient as described (2). Four-plex dPCRs were performed in separate reactions for graft and recipient. Molecule concentrations were calculated by Poisson statistics using molecule counts of four combined graft-specific dPCRs with 5-7µL sample volume and one recipient-specific reaction (µL sample). Simulations were computed to assess the achievable total precision of dPCR quantifications with and without preamplification. These results were experimentally validated by direct comparison of both methods in 11 cfDNA pools of mixed normal and 19 transplant patient samples containing 0.1%-30% GcfDNA. Twenty-four four-plex dPCRs were performed in 207 HTx patient samples.

Results:

The ligation efficiency is the limiting step in preamplification and ranges from 40% to 85%. In silico simulations, considering this ligation variability and the low DNA amount, indicated that direct multiplex quantification is superior to dPCR with preamplification, despite 40-fold higher positive events in the latter. Consistently, the relative standard deviation (CV) in 11 cfDNA pools was 2.1-fold higher after preamplification. The CVs of the multiplex method in 19 HTX patient samples (mean:11%;SD:6,6%) were significantly lower than those obtained with preamplification (mean:28%;SD:15%, p=0.009). In 207 HTx patient samples with a median total cfDNA concentration of 63,210cp/mL plasma (range:2,415-2,102,861) the median GcfDNA was 0.57% (range:0.02-12.1%) resulted in a median CV of 7.6% (range:1.0-38.3). A CV of <30% was achieved in 98.5% of all patient samples and 90% yielded <20% CV; the GcfDNA in the latter group ranged from 0.03-12.1% and 21-43,530cp/mL plasma.

Conclusions:

The reliable quantification of rare DNA moieties is technically challenging, especially in cfDNA, with the scarce total DNA amount. The use of dPCR or HTS eliminates a potential calibration bias by direct molecule counting. However, the hypergeometric contribution to the total measurement error, the variability in preamplification is deteriorating the precision, especially if the number of targets is very low, as in GcfDNA in HTXs. Since this is a random error, it cannot be eliminated and therefore, avoiding preamplification resulted in lower imprecision. The advantage of direct cfDNA molecule counting is the ability to assess the total error by the Poisson counting error. With the testing of four independent SNPs in one dPCR and the summation of five multiplex dPCRs into one final result the number of counted molecules is sufficiently high to quantify GcfDNA levels in HTx patients with high precision at lower costs compared to HTS based methods.


Problem description

dPCR “Goldilocks Zone”

- Very high and very low proportion of positive PCR reactions give increased uncertainty
- Uncertainty is lowest in the middle

Multiplexing strategy

Four different assays were combined in multiplex ddPCRs and graft and recipient alleles were measured in separate reactions, each reaction contains only the recipient-specific or the graft-specific probes, as exemplified in the table. Graft-specific reactions contained 5-7µL extracted cfDNA, while recipient-specific reactions contained only 1µL. Graft-specific alleles were summed from 4 independent reactions and the mean number of template molecules per droplet and the respective error were calculated based on Poisson statistics. The GcfDNA fraction is then calculated using the mean concentrations of graft and recipient.

SNP-Assay

Recipient Genotype and Probe

Graft Genotype and Probe

1
AA – Probe A
BB – Probe B
2
BB – Probe B
AB – Probe A
3
AA – Probe A
BB – Probe B
4
BB – Probe B
AA – Probe A

Results - Simulation

The ligation efficiency is the limiting step in preamplification and ranges from 40% to 85%. In silico simulations, considering this ligation variability and the low DNA amount, indicated that direct multiplex quantification is superior to dPCR after preamplification, despite 40-fold higher positive events that can be achieved in the latter.

Results - Patient Data

In 207 HTx patient samples with a median total cfDNA concentration of 63,210cp/mL plasma (range:2,415-2,102,861) the median GcfDNA was 0.57% (range:0.02-12.1%) resulted in a median CV of 7.6% (range:1.0-38.3). A CV of <30% was achieved in 98.5% of all patient samples and 90% yielded <20% CV; the GcfDNA in the latter group ranged from 0.03-12.1% and 21-43,530cp/mL plasma.

All patient samples were drawn under IRB-approved informed consent.

Transplant Liquid Biopsy Test

Healthy

1. Collect cell-free DNA from recipients plasma
2. Identify informative SNPs out of a preselected set of 38 assays
3. Quantify graft-derived cell-free DNA by droplet digital PCR (ddPCR)

Organ damage

Method published in

Beck J., Bierer S., Balzer S., et al.,