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# Probe-Free Digital PCR Quantitative Methodology to Measure Donor-Specific Cell-Free DNA after Solid-Organ Transplantation

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**BACKGROUND:** Donor-specific cell-free DNA (dscfDNA) is increasingly being considered as a noninvasive biomarker to monitor graft health and diagnose graft rejection after solid-organ transplantation. However, current approaches used to measure dscfDNA can be costly and/or laborious. A probe-free droplet digital PCR (ddPCR) methodology using small deletion/insertion polymorphisms (DIPs) was developed to circumvent these limitations without compromising the quantification of dscfDNA. This method was called PHABRE-PCR (*P*rimer to *H*ybridize across an *A*llelic *BRE*akpoint-PCR). The strategic placement of one primer to hybridize across an allelic breakpoint ensured highly specific PCR amplification, which then enabled the absolute quantification of donor-specific alleles by probe-free ddPCR.

**METHODS:** dscfDNA was serially measured in 3 liver transplant recipients. Donor and recipient genomic DNA was first genotyped against a panel of DIPs to identify donor-specific alleles. Alleles that differentiated donor-specific from recipient-specific DNA were then selected to quantify dscfDNA in the recipient plasma.

**RESULTS:** Lack of amplification of nontargeted alleles confirmed that PHABRE-PCR was highly specific. In recipients who underwent transplantation, dscfDNA was increased at day 3, but decreased and plateaued at a low concentration by 2 weeks in the 2 recipients who did not develop any complications. In the third transplant recipient, a marked increase of dscfDNA coincided with an episode of graft rejection.

**CONCLUSIONS:** PHABRE-PCR was able to quantify dscfDNA with high analytical specificity and sensitivity. The implementation of a DIP-based approach permits surveillance of dscfDNA as a potential measure of graft health after solid-organ transplantation.



Surveillance of graft health after solid-organ transplantation remains a fundamental aspect of posttransplantation care. The definitive evaluation of graft health is based on the histological assessment of tissue biopsies. These carry significant risks of complications, including pain, bleeding, and sepsis *(1, 2)*. Furthermore, the accuracy of tissue biopsies is often challenged by sampling errors and interpathologist variation *(3–5)*. Due to the invasive nature of biopsies, and the lack of clinical specificity and sensitivity of routine serum biomarkers, considerable efforts have been made to develop accurate noninvasive biomarkers to monitor graft health *(6–9)*.

Release of cell-free DNA is thought to arise following cellular apoptosis and necrosis *(10, 11)*. Since the discovery of donor-specific cell-free DNA (dscfDNA)[5] *(12)*, there has been substantial interest in the study of dscfDNA as a biomarker to monitor graft health *(13, 14)* and diagnose graft rejection *(15, 16)*. In episodes of increased cell death following graft injury (i.e., ischemic injury, immunity-mediated rejection, or sepsis), an increased concentration of dscfDNA is thus quantifiable in the circulation of the recipient.

The detection of dscfDNA is reliant on first identifying distinct genetic differences between the donor and the recipient. The presence of the Y chromosome (derived from a male donor organ) in the circulation of a female recipient can be used to differentiate donor- and recipient-specific DNA. However, the use of this ap-

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[5] Nonstandard abbreviations: dscfDNA, donor-specific cell-free DNA; SNP, single-nucleotide polymorphism; DIP, deletion/insertion polymorphism; ddPCR, droplet digital PCR; PHABRE-PCR; *P*rimer to *H*ybridize across an *A*llelic *BRE*akpoint-PCR; HRMA, high resolution melting analysis.

proach limits the surveillance of female recipients with organs derived from male donors *(14, 17–19)*.

Genetic polymorphisms like single-nucleotide polymorphisms (SNPs) or deletion/insertion polymorphisms (DIPs) are effective markers to distinguish a chimeric mixture of donor- and recipient-specific DNA. Allelic sequences of a polymorphic locus that are only present in the donor, and absent in the recipient, are considered informative and will differentiate donor-specific DNA from recipient-specific DNA. The use of an appropriately sized panel of genetic polymorphisms with high heterozygosity will enable the monitoring of all transplantation cases.

Several approaches using genetic polymorphisms have been reported. The measurement of dscfDNA can be performed by real-time quantitative PCR using large DIPs to discriminate donor and recipient DNA *(13, 20)*. However, absolute quantification by standard-curve calibration can be imprecise in the setting of low abundance dscfDNA *(21, 22)*.

The quantification of dscfDNA can also be performed by massively-parallel sequencing of plasma to identify donor- and recipient-specific SNPs *(15, 16, 23)*. However, sequencing-based approaches are expensive, laborious, analytically less sensitive, and generally have an unacceptable turnaround time.

The advent of droplet digital PCR (ddPCR) has enabled the quantification of rare DNA targets with unprecedented accuracy by limiting dilution *(24–26)*. Hence, the use of ddPCR is ideal to monitor the dynamic changes in dscfDNA after transplantation. The use of ddPCR to monitor dscfDNA after transplantation was first demonstrated by Beck and colleagues *(27)*, who used PCR preamplification followed by probe-based assays to detect donor- and recipient-specific SNPs and quantify dscfDNA concentrations. However, the use of PCR preamplification is an additional step that may introduce biases and thus confound downstream quantitative analyses. In addition, the use of probes adds to the complexity and cost of the assays.

We describe a novel ddPCR approach that is probe-free and does not require PCR preamplification to measure dscfDNA concentrations. Small DIPs (between 10 and 50 bp) were selected in our approach since such markers facilitated (*a*) the rapid genotyping of donor and recipient blood samples *(28)* and (*b*) the absolute quantification of dscfDNA using PHABRE-PCR (*P*rimer to *H*ybridize across an *A*llelic *BRE*akpoint-PCR), a technique based on the placement of a primer that hybridized across an allelic breakpoint, enabling specific amplification of donor-specific alleles (Fig. 1).

## Methods and Materials

### STUDY PARTICIPANTS

Ethical approval for this study was obtained from the Australian Red Cross Blood Service (agreement number 15-06VIC-07), Donate Life, Australia (project number 2015#04), and the Austin Health Human Research Ethics Committee (reference number HREC/15/Austin/142). Written informed consent was obtained before the collection of blood samples. Genomic DNA from these individuals was extracted from leukocyte-rich fraction of the blood samples using the QIAamp DNA Mini Kit (Qiagen).

### DELETION AND INSERTION POLYMORPHISMS

A panel of 9 small biallelic DIPs were selected using the Marshfield Clinic database *(29)*. Six of the DIPs were reported in our previous publication *(28)*. Supplemental Table 1 that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue3 summarizes the characteristics of the polymorphisms. Primer sets used in the genotyping of each DIP by high-resolution melting analysis (HRMA) are summarized in online Supplemental Table 2.

Nineteen healthy individuals were genotyped by HRMA for the DIPs to determine the melting curves for 3 genotypes (insertion/insertion, deletion/deletion, or deletion/insertion) for each locus. HRMA was performed as previously described *(28)*.

### DESIGN OF PHABRE-PCR PRIMER SETS

The placement of one primer across an allelic breakpoint enabled the specific amplification of the DIP allele of interest by PHABRE-PCR. Since the allelic breakpoint of a DIP locus with the insertion allele comprises 2 junctions at either end of the insertion, a primer that spans across one insertion junction (or both insertion junctions in the case of smaller DIPs) enables specific amplification of the insertion allele in combination with a common primer (Fig. 1). Conversely, the allelic breakpoint of a DIP locus with the deletion allele comprises a single deletion junction, hence a primer that spans the deletion junction will only amplify the deletion allele in combination with a common primer.

Primer sets were designed to produce small amplicons (between 50 and 130 bp) to maximize the detectable number of dscfDNA templates. The details of the 18 primer sets that were designed to either amplify the long and short alleles of each DIP locus are summarized in online Supplemental Table 3.

### STEP 1 (GENOTYPING BY HRMA)

Blood samples from 3 deidentified, deceased organ donors and matched transplant recipients were obtained before transplantation. Genomic DNA was genotyped against the panel of 9 DIPs by HRMA *(28)*. The DIP was considered informative if an allele was present in the donor and absent in the recipient (see online Supplemental Table 4).

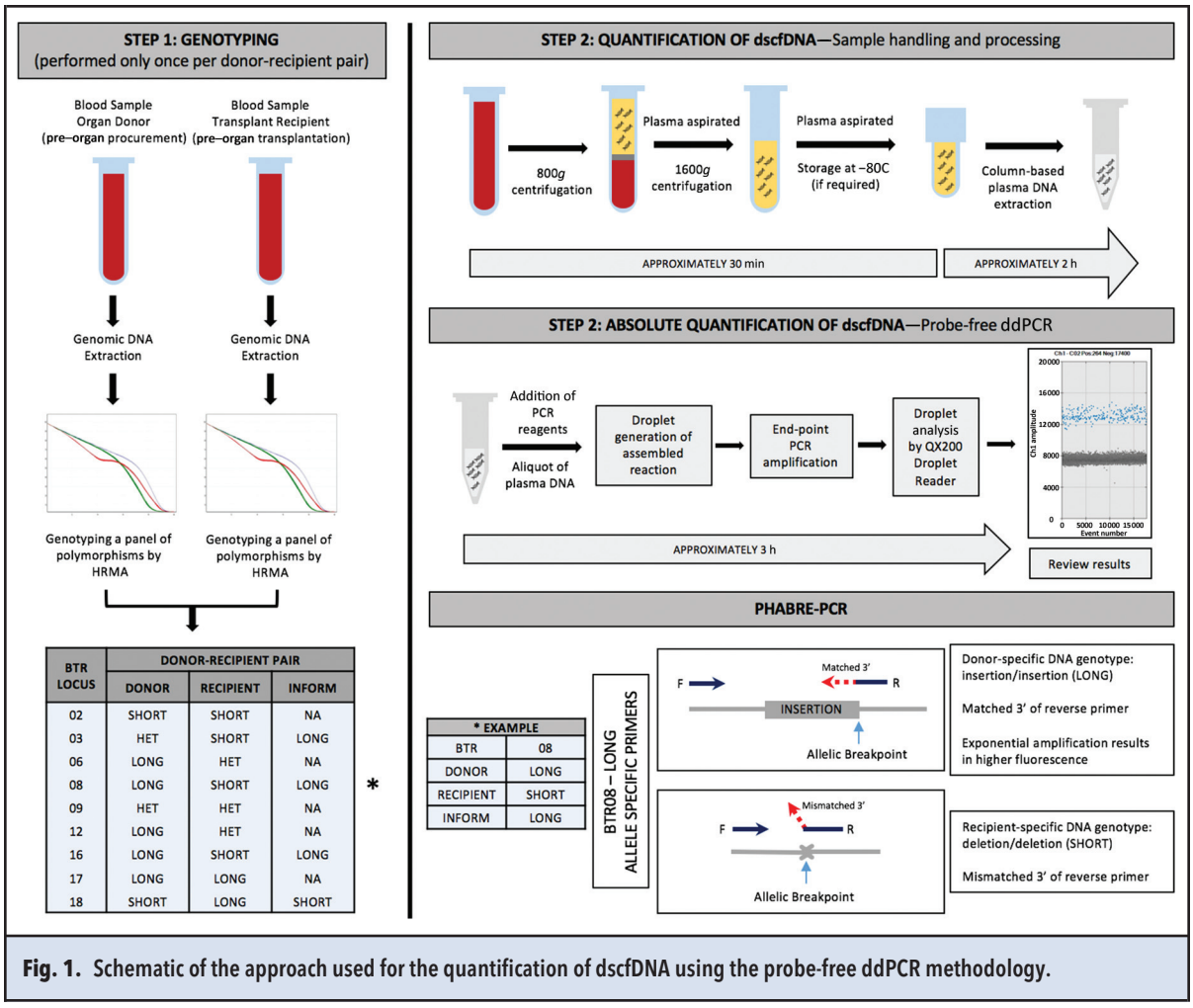


**Fig. 1.** Schematic of the approach used for the quantification of dscfDNA using the probe-free ddPCR methodology.

### STEP 2 (QUANTIFICATION OF dscfDNA)

This step allowed quantification of dscfDNA using the informative alleles established in Step 1. Dependent on the informative allele, the corresponding PHABRE-PCR assay was selected to quantify dscfDNA (see online Supplemental Table 3).

Eighteen milliliters of blood from each transplant recipient was collected using VACUETTE® potassium-EDTA blood collection tubes (Greiner Bio-One International). Each sample was processed within 3 h of collection. The following time points were used at pretransplant and at posttransplant days 3, 7, 14, 28, and 42. By using previously described protocols *(30)*, each blood sample was centrifuged at 800*g* for 10 min. The plasma fraction of the blood sample was transferred into a collection tube for a second centrifugation step at 1600*g* for 10 min to minimize cellular contamination. Subsequently, the plasma was aspirated and transferred into cryovials (Corning) for storage at −80 °C.

Plasma samples were analyzed in batches. Four milliliters of plasma from the transplant recipients was thawed to ambient temperature, and plasma DNA was extracted using the QIAamp Circulating Nucleic Acid Kit (Qiagen) DNA. All samples were eluted in 100 μL of AVE buffer and kept at 4 °C.

Informative donor alleles were selected to serially quantify dscfDNA concentrations for each transplant recipient after genotyping. In brief, a 22 μL reaction comprising 1× QX200 ddPCR EvaGreen Supermix (Bio-Rad), 100 nmol/L of each forward and reverse primer, 2 μL of plasma DNA, and PCR-grade water was prepared. Twenty microliters from each ddPCR mix was loaded onto the DG8 droplet generator cartridge (Bio-Rad) for droplet generation. After partitioning, the reactions were cycled on a C1000 Touch thermocycler (Bio-Rad) using the following conditions: one cycle of 95 °C for 5 min; 40 cycles of 95 °C for 30 s and 61 °C for 60 s; 1 cycle of 4 °C for 5 min; 1 cycle of 90 °C for 5 min and a brief hold at 4 °C.

After the end-point PCR, the 96-well plate was analyzed using a QX200 Droplet Reader (Bio-Rad). By using the supplied software, QuantaSoft (Bio-

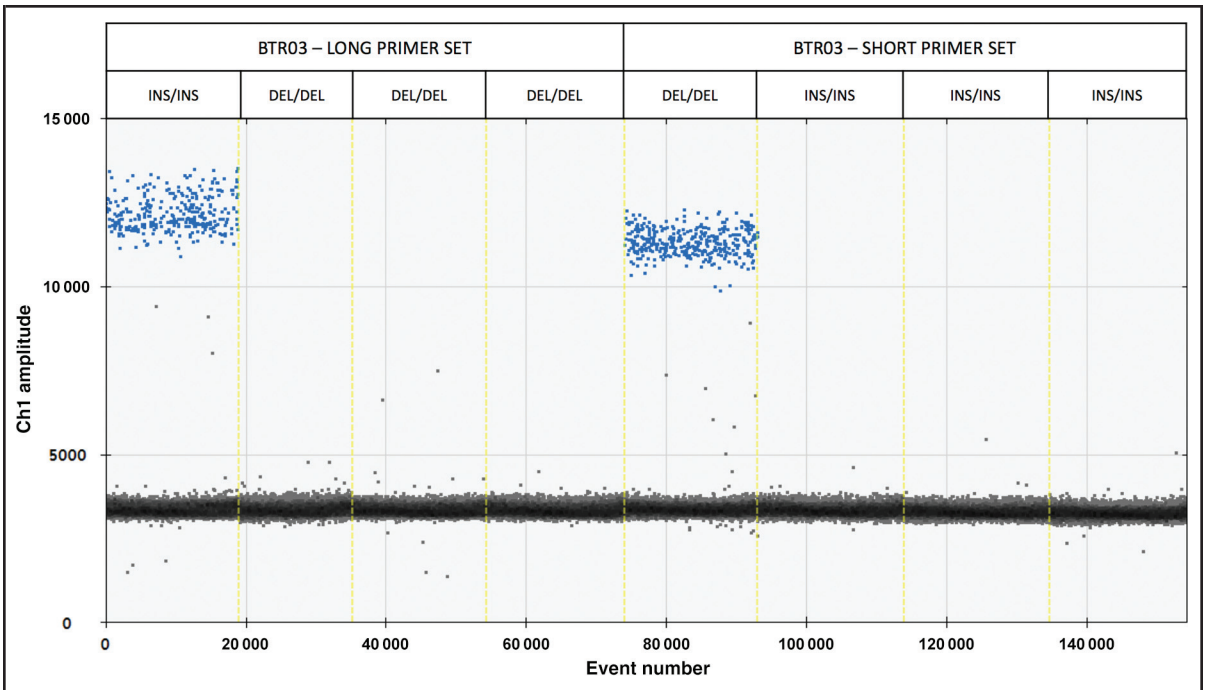


**Fig. 2. Analytical specificity of PHABRE-PCR for the 2 alleles of BTR03.**

Blue droplets indicate amplified templates. Grey droplets indicate nonamplifiable templates. Lane 1: the long (insertion allele) primer set amplified the genomic DNA with an insertion/insertion (INS/INS) genotype. Lanes 2, 3, and 4: the long (insertion allele) primer set did not amplify the genomic DNA with a deletion/deletion (DEL/DEL) genotype. Lane 5: the short (deletion allele) primer set amplified the genomic DNA with a DEL/DEL genotype. Lanes 6, 7, and 8: the short (deletion allele) primer set did not amplify the genomic DNA with INS/INS genotypes.

Rad), absolute quantification using the EvaGreen chemistry was selected. The derived concentration was normalized to dscfDNA copies per mL of recipient plasma (presented in this study as copies/mL) by the following formula:

$$\frac{\text{copies}}{\text{mL}} = \left( x \frac{\text{copies}}{\mu\text{L}} \right) \times \left( \begin{matrix} 22\ \mu\text{L of assembled} \\ \text{ddPCR reaction} \end{matrix} \right) \times \left[ \frac{\dfrac{100\ \mu\text{L}}{y\ \mu\text{L}}\ \text{of plasma DNA eluate}}{\begin{matrix} z\ \text{mL of plasma} \\ \text{used for extraction} \end{matrix}} \right], \quad (1)$$

where $x$ = copies of dscfDNA/$\mu$L of the assembled PCR reaction as calculated by the QuantaSoft software, $y$ = $\mu$L plasma DNA eluate used for ddPCR reaction, and $z$ = mL plasma used for extraction of plasma DNA.

Since the gene dosage per cell for a homozygote allele was double that of a heterozygote allele, the concentration of positive droplets from homozygote donor-specific loci was divided by 2 to normalize genomic content.

## Results

### UNIQUE MELTING PROFILE FOR EACH DIP

We previously published the genotype-specific melting profiles for 6 DIP loci *(28)*. In the study reported here, we evaluated 3 additional DIPs and the 3 genotype-specific melting profiles for each DIP were established. The genotype-specific melting profiles of our panel of 9 DIPs are summarized in online Supplemental Fig. 1. Genotypes of the 3 matched donor-recipients were determined by comparison with known genotype-specific melting profiles.

### EVALUATING THE COVERAGE OF THE PANEL OF 9 DIPs

The coverage of the panel of 9 DIPs was evaluated using the genotypes of 25 individuals (comprising 19 healthy individuals, and the 3 donors and 3 recipients that were recruited in this study). The individuals were cross-matched against each other to generate a combination of 600 potential donor-recipient pairs. With the use of our panel of 9 DIPs, at least 1 informative allele was identified in 589 (98.2%) of the potential donor-recipient pairs.

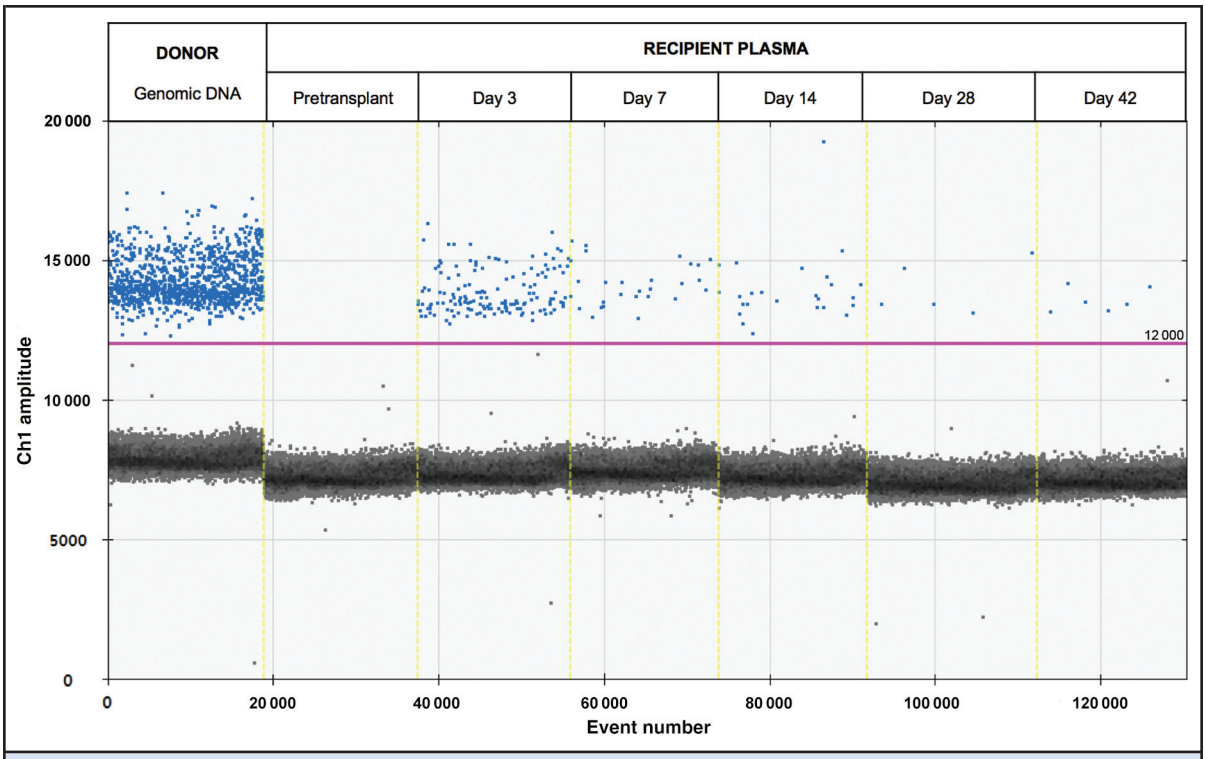


**Fig. 3. A representative probe-free ddPCR 1D plot derived from a PHABRE-PCR assay for the absolute quantification of dscfDNA.**
BTR16 – LONG was used to quantify dscfDNA in recipient LT2 who underwent liver transplantation without any complications. The assay amplifies the positive control (genomic DNA from the buffy coat of the matched donor recipient). dscfDNA concentrations were analyzed at pretransplant through posttransplant day 42. The threshold was selected at 12 000 relative fluorescent units to assign positive (blue) droplets containing dscfDNA. Grey droplets indicate droplets that did not contain any amplifiable templates.

### ANALYTICAL SPECIFICITY OF PHABRE-PCR

The analytical specificity was assessed for both the long and short alleles of each DIP locus. Both the long and short primer sets were used to amplify DNA with an insertion/insertion genotype. Both primer sets were also used to amplify DNA with a deletion/deletion genotype. All loci performed consistently. The lack of nonspecific amplification of short alleles using the long allele primer sets, and vice versa confirmed the analytical specificity of our approach. Fig. 2 shows BTR03 as a representative locus (3 other loci are presented in online Supplemental Fig. 2, a–c).

### RECIPIENTS WITHOUT POSTTRANSPLANT COMPLICATIONS

Donor and recipient pairs (LT1 and LT2) were genotyped by HRMA (see online Supplemental Table 5). The genotype for each DIP was determined by comparison with known controls for genotype-specific melting profiles in the same run. The donor for recipient LT1 had 3 informative alleles and the assays designed to amplify the long alleles of BTR16 and BTR18 were selected to quantify dscfDNA. The donor for recipient LT2 had 1 informative allele and the assay designed to amplify the long allele of BTR16 was selected to quantify dscfDNA.

As shown in Fig. 3, positive droplets (containing template, in this case dscfDNA) clustered at a markedly higher fluorescent intensity compared to the negative droplets (containing no amplifiable template). PHABRE-PCR showed minimal "rain" (droplets with fluorescence ranging between explicitly positive and negative droplets). The analytical specificity of PHABRE-PCR was also confirmed by the lack of dscfDNA amplification in the pretransplant samples.

The quantification of dscfDNA demonstrated similar trends in the decrease of dscfDNA concentrations in 2 recipients who did not develop posttransplant complications (Fig. 4). There was a marked reduction in dscfDNA from day 3 to day 7. The dscfDNA plateaued at a low concentration from day 7 onward. This finding was also consistent with the pattern of improvement in serum liver function tests after transplantation in both recipients LT1 and LT2 (represented by recipient LT1 in Fig. 5).

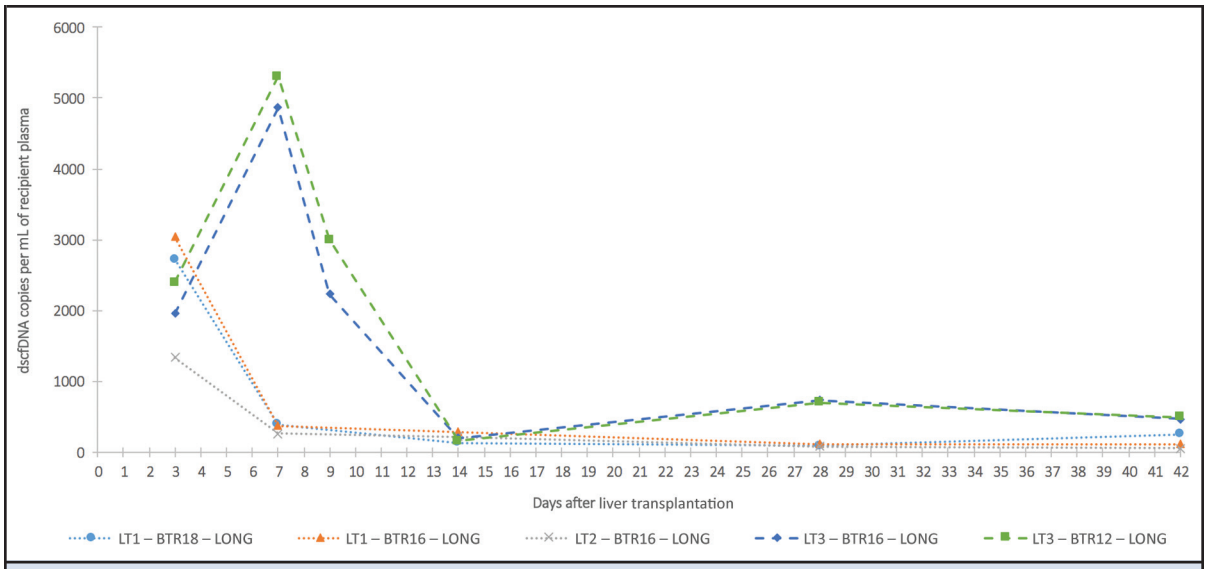


**Fig. 4. Donor-specific cell-free DNA (dscfDNA) concentrations in 3 transplant recipients.**

Recipients LT1 and LT2 underwent transplantation without any transplant complications. The marked rise in donor-specific cell-free DNA concentrations in recipient LT3 on day 7 is attributable to the development of graft rejection. The concentrations decreased following an adjustment in immunosuppressive therapy in this particular recipient.

### ONE RECIPIENT WHO DEVELOPED GRAFT REJECTION

Genotyping of recipient LT3 identified 5 donor-specific informative loci (see online Supplemental Table 5). The long alleles of BTR12 and BTR16 were selected to monitor the dscfDNA.

Recipient LT3 developed an episode of graft rejection (specifically acute cellular rejection) at day 7. Abnormal liver function tests prompted a liver biopsy that confirmed the diagnosis. Following the adjustment of immunosuppression, the serum liver function tests showed subtle improvements.

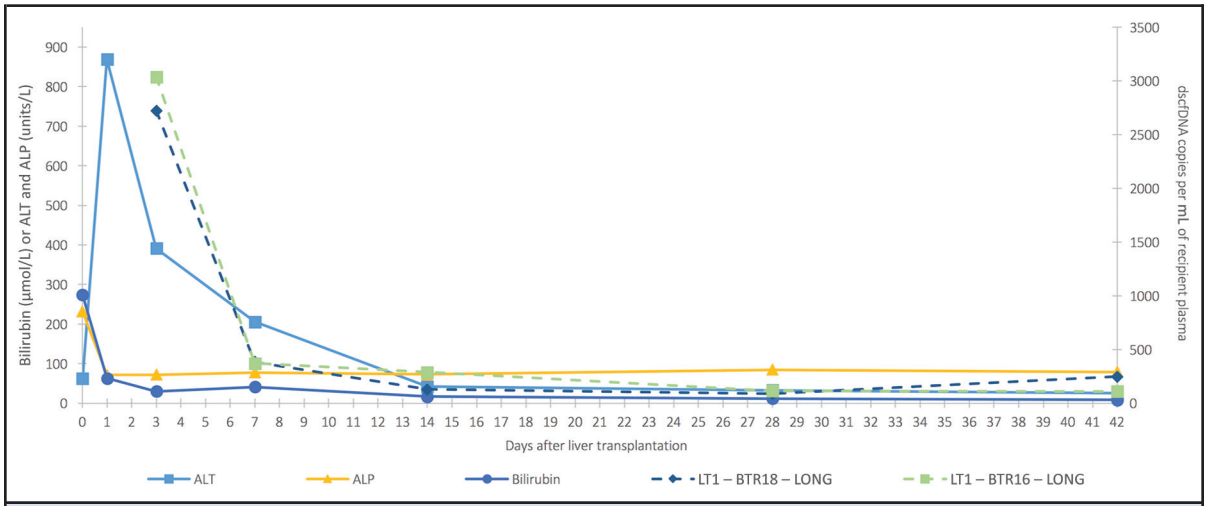


**Fig. 5. Serum liver function tests and dscfDNA concentrations of recipient LT1 who underwent liver transplantation without any posttransplant complications.**

dscfDNA concentrations were quantified using PHABRE-PCR assay BTR18 – LONG and BTR16 – LONG. ALT, alanine aminotransferase; ALP, alkaline phosphatase.

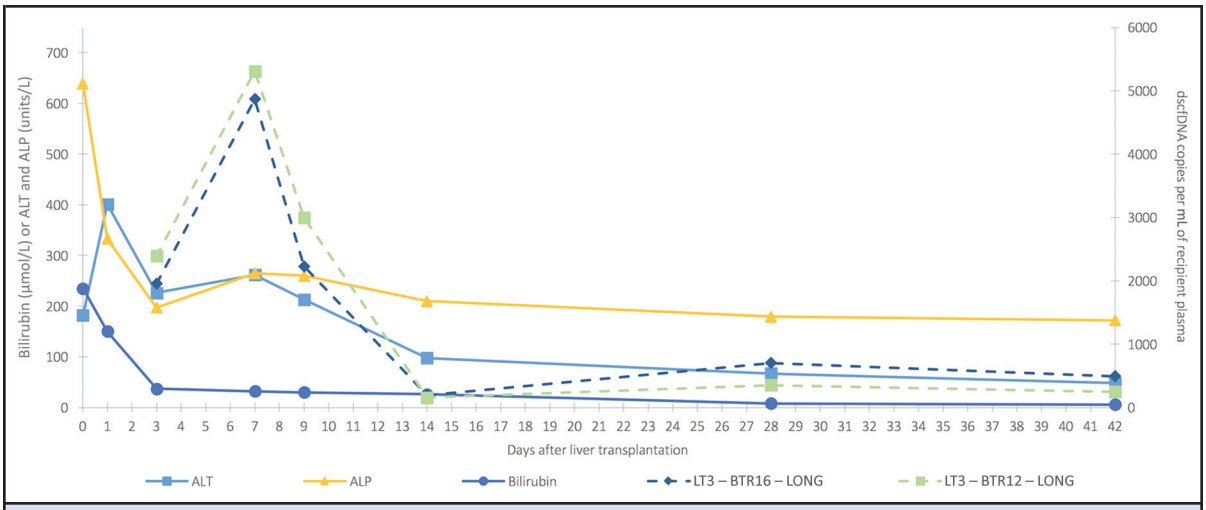


**Fig. 6. Serum liver function tests and dscfDNA concentrations of recipient LT3.**

The recipient developed an episode of graft rejection at day 7. A liver biopsy was performed to investigate the persistent elevation of alanine aminotransferase (ALT) and alkaline phosphatase (ALP). Following the diagnosis, immunosuppressive therapy was increased. DscfDNA concentrations in recipient LT3 were measured using PHABRE-PCR assays BTR16 – LONG and BTR12 – LONG. The increase of dscfDNA correlated with the diagnosis of biopsy-proven acute cellular rejection and decreased with the adjustment of immunosuppressive therapy.

In contrast, dscfDNA provided a superior indication of the compromised graft health compared to serum liver function tests in this particular recipient (Fig. 6). On day 3, dscfDNA concentrations in recipient LT3 were similar to those in the healthy recipients. However, in the blood sample taken on the day before a liver biopsy that was performed to confirm graft rejection, the dscfDNA concentrations were markedly increased (BTR12 – LONG: 5308 copies/mL and BTR16 – LONG: 4868 copies/mL). The adjustment of immunosuppression resulted in an improved graft health. This was reflected by the decrease of dscfDNA concentrations on day 9 (BTR12 – LONG: 2998 copies/mL and BTR16 – LONG: 2228 copies/mL) and day 14 (BTR12 – LONG: 155 copies/mL and BTR16 – LONG: 197 copies/mL).

However, the dscfDNA concentrations of recipient LT3 remained increased compared to the other 2 recipients at day 28 and day 42 without any evidence of clinically compromised graft function (Fig. 4). Despite the notable difference in copy number between the 2 quantified alleles (BTR 12 – LONG and BTR16 – LONG), the correlation ($R^2$) between the 2 alleles was 0.98.

## Discussion

There is increasing interest in dscfDNA as a noninvasive biomarker of organ health and organ rejection. The methodologies used to date have intrinsic limitations that may preclude their implementation into clinical practice. We believe that our approach can overcome many of these limitations and facilitate its translation into the diagnostic laboratories.

We performed the required first step of genotyping a set of DIPs on pretransplantation blood samples by HRMA. As previously noted, HRMA is not only accurate and rapid, HRMA-capable PCR platforms are also widely available *(28)*. Furthermore, HRMA-based genotyping eliminates the need for the more complex methodologies previously reported, i.e., massively parallel sequencing techniques *(15, 16, 23)*, techniques that require fluorescent probes for PCR-based genotyping *(27)*, and/or electrophoretic fragment length analysis that can be prone to error and carry the risks of PCR-product contamination *(13, 20)*.

Although the panel of DIPs achieved a comprehensive coverage of potential donor-pair recipients, there will be a small subset of the donor-recipient pairs in which an informative allele will not be identified. The use of additional DIP-based assays can overcome this limitation.

ddPCR offers the technical advantages of reproducibility *(22)* and absolute quantification without the need of standard-curve calibration of real-time quantitative PCR *(13, 20)*. This technology is therefore attractive for the monitoring of dscfDNA after transplantation.

The novelty of PHABRE-PCR is based on the design of primer sets, in which one of the primers of an allele-specific primer set hybridizes across the allelic breakpoint of an informative DIP to selectively amplify donor alleles (Fig. 1). In this study, PHABRE-PCR was highly specific, which enabled the detection of donor-

specific alleles in the recipient plasma and absolute quantification of dscfDNA (Fig. 2 and 3).

The use of EvaGreen chemistry on a ddPCR platform eliminates the need for costly fluorescent probes *(20, 27)*. Since probe-based assays often require meticulous optimization and may have problems with cross-reactivity (necessitating redesigns), a probe-free approach is more economical and straightforward.

Importantly, the methodology employed in this study to quantify dscfDNA did not use a PCR preamplification step. PCR preamplification will affect allelic quantification, as well as requiring more manual handling and carrying a considerable risk of contamination.

The current lack of standardization in the measurement of dscfDNA has been reviewed by Gielis et al. *(31)*. dscfDNA is frequently measured based on the relative abundance of donor DNA (percentage of donor DNA = donor DNA divided by sum of donor and recipient DNA) *(15, 23, 27)*. However, calculations that include the concentration of recipient DNA have inherent limitations. Studies have shown that the improper handling of blood samples as well as infection and exercise can nonspecifically increase recipient DNA *(32–34)*, and these factors are likely to confound the measurement of relative abundance. As such, only absolute measurements of dscfDNA related to the initial plasma volume are presented in this study.

Although it is challenging to directly compare the dscfDNA concentrations of prior studies that used real-time PCR for absolute quantification, *(14)* or digital PCR for relative abundance quantification *(27)*, our findings support the notion that the observation of dscfDNA at a low concentration after transplantation is generally reflective of a healthy graft.

Marked increases in dscfDNA concentrations were observed in 1 recipient and this correlated with an episode of biopsy-proven graft rejection at day 7. This finding was consistent with the current published literature. In a large prospective study comprising 65 heart transplant recipients, an increased concentration of dscfDNA in recipients was diagnostic of graft rejection *(16)*. In a smaller study comprising 17 liver transplant recipients, increased dscfDNA was also found to be associated with graft rejection *(27)*. Nevertheless, the absolute quantification of dscfDNA in our study effectively monitored the clinical progress of the same recipient using 1 or more donor-specific alleles after liver transplantation.

## Conclusion

We assessed the use of PHABRE-PCR for absolute quantification of dscfDNA. As proof-of-principle, PHABRE-PCR enabled the measurement of dscfDNA concentrations in 3 recipients who underwent liver transplantation. Compared to sequencing and other PCR-based quantitative methodologies, PHABRE-PCR is a relatively inexpensive, yet accurate, probe-free digital PCR methodology to measure dscfDNA after transplantation.

**Author Contributions:** *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

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