Clinical Chemistry 59:12
1732–1741 (2013)

Digital Droplet PCR for Rapid Quantification of Donor DNA in the Circulation of Transplant Recipients as a Potential Universal Biomarker of Graft Injury

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BACKGROUND: Cell-free DNA (cfDNA) from grafts in the circulation of transplant recipients is a potential biomarker of rejection. Its usefulness was investigated after heart transplantation during the maintenance phase by use of microarrays and massive parallel sequencing of donor and recipient DNA. Disadvantages of these methods are high costs, long turnaround times, and need for donor DNA. Therefore, we sought to develop a rapid and cost-effective method using digital droplet PCR (ddPCR).

METHODS: Plasma samples were collected from stable recipients after liver (LTx, n = 10), kidney (KTx, n = 9), and heart (HTx, n = 8) transplantation as well as from 7 additional patients directly after LTx. Known single-nucleotide polymorphisms were selected for high minor allelic frequencies, of which 41 hydrolysis probe assays were established. Plasma cfDNA was preamplified, followed by conventional real-time PCR to define informative (heterologous) SNPs, which were then used for quantification (percentage) of graft-derived cfDNA (GcfDNA) using ddPCR.

RESULTS: Mean recovery was 94% (SD, 13%) with an imprecision of 4%–14% with the use of controls with 2% minor allele. GcfDNA in stable patients was <6.8% (LTx), <2.5% (KTx), and <3.4% (HTx). On the day of LTx, GcfDNA was approximately 90% and by day 10 it was <15% in complication-free LTx recipients. In 2 patients with biopsy-proven rejection, GcfDNA increased to >60%, whereas in 1 patient with cholestasis no increase was found.

CONCLUSIONS: A novel, cost-effective, rapid technique was developed to quantify GcfDNA in transplant recipients. This technique embodies a promising, potentially universal biomarker for early detection of rejection, which could enable more effective therapeutic interventions.

The detection of trace amounts of variants in genetic material in a single sample is feasible with the use of advanced molecular biology techniques and has several potential clinical applications, including the identification of transplant rejection *(1)*. An increase of heart donor DNA in the circulation of stable heart transplant recipients from values of <1% to values up to 5% during rejection episodes has been reported *(2)*. However, to be clinically useful the method used for the detection of graft DNA must not only be specific and sensitive, it must also have a rapid turnaround time and be economically feasible to perform. Some methods described to date are extremely time-consuming and expensive to perform *(3)*, or they can be used only in male-to-female engrafting *(4)*.

Simpler methods to differentiate between DNA from donors and recipients can involve the use of single-nucleotide polymorphisms (SNPs).[5] One possibility is to interrogate both donor and recipient for certain SNPs and use those for which both SNPs are homozygous, but different in donor and recipient. However, this would require DNA from the donor to be available, which is not always possible in the clinical setting, particularly if the transplantation was performed some years before. One way to overcome this need is to use SNPs that have been investigated for their minor allelic frequency (MAF) and show frequencies higher than, e.g., 0.4. Assuming Hardy–Weinberg equilibrium, an SNP with an MAF between 0.4 and 0.5 would be found homozygous in both donor and recip-

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Received May 29, 2013; accepted August 19, 2013.
Previously published online at DOI: 10.1373/clinchem.2013.210328
[5] Nonstandard abbreviations: SNP, single-nucleotide polymorphisms; MAF, minor allelic frequency; cfDNA, cell-free DNA; ddPCR, digital droplet PCR; GcfDNA, graft-derived cfDNA; LTx, liver transplantation; KTx, kidney transplantation; HTx, heart transplantation; AST, aspartate aminotransferase; GGT, γ-glutamyltransferase.

ient 23% to 25% of the time for each allele. The probability of both donor and recipient having a different allele is therefore 11.5% to 12.5%, using accepted estimation models for calculation of exclusion probabilities *(5)*. To identify at least 3 such SNPs in white individuals, no fewer than 30–35 different SNPs with the mentioned characteristics should be interrogated. For comparison, if unselected SNPs were to be used, it can be estimated that over 3000 assays would be needed to achieve the same discriminating power, based on the median MAF of approximately 0.023 reported for known human SNPs (cf., Illumina HumanOmni5M SNP Beadchip).

Using such SNP comparisons, the amount of graft DNA released by an organ into the circulation can be assessed and used as a biomarker for organ integrity. In addition, once SNPs that differ between donor and recipient are identified, only SNPs with the best sensitivity (homozygous in both but different in donor and recipient) need to be measured subsequently. The only limitation of such a method is the amount of DNA that is interrogated, which is mainly driven by the volume of blood that is analyzed. It has been estimated that the number of genome equivalents in 1 mL of blood is about 1000 *(6, 7)*. Assuming the graft DNA accounts for 5% of cell-free DNA (cfDNA), if all molecules take part in a PCR reaction there would be 50 such molecules in 1 mL of blood. The entire analysis of several different SNPs, therefore, requires as a first step the random unbiased amplification of extracted cfDNA from not less than 2 mL of blood. Although such an amplification step can be performed by several techniques, for the usually short apoptotic cfDNA *(8)* a direct adaptor ligation is most suitable *(9)*. Once the amplification adaptors are ligated, a moderate number of amplification cycles is added (not more than 10–12), and the resulting library following removal of primers and adaptors is used as the template for the SNP interrogations mentioned above. We developed a novel digital droplet PCR (ddPCR) technique enabling rapid, cost-effective, and precise quantification of graft cfDNA at low abundance and applied this method for the first time to quantify graft-derived cfDNA (GcfDNA) following solid organ transplantation.

Material and Methods

PATIENTS

Patients immediately after liver transplantation (LTx) (n = 8 with 1 dropout, from whom no samples could be obtained) and from outpatients during the stable maintenance phase (>6 months) after LTx (n = 10), heart transplantation (HTx) (n = 8), and kidney transplantation (KTx) (n = 9) were included in the study. For assay development and evaluation of assay reproducibility, DNA from healthy volunteers (n = 6) was used. Samples were drawn under an institutional review board–approved protocol with informed consent, where applicable.

SNP HYDROLYSIS ASSAYS

SNPs were selected from different compilations of public databases (e.g., Hapmap or 1000Genomes), considering those which show a known and validated MAF of >43% in whites and over all reported ethnicities. As a next step, SNPs in or directly adjacent to a repetitive element were eliminated. The remaining SNPs were then investigated for their usefulness in a probe hydrolysis assay. This was done in silico by using thermodynamic calculations *(10)* to optimize the binding differences for the 2 probes that hybridize to the 2 alleles at the desired temperature of 65 °C at standard PCR buffer conditions (e.g., 0.18 mol/L monovalent cations and 0.5 μmol/L DNA/Primer). Because the slope of a double-stranded DNA probe melting curve is mainly dependent on the enthalpy *(11)* of the probes, the enthalpy dominates the selection for a maximized difference of free Gibbs energy between allele binding at a given condition. We designed 41 probe sets using different probes for each of the 2 alleles; the fluorescent dyes FAM and HEX were used in conjunction with BHQ1 as quencher (MWG-Biotech). Using the conditions given above and published formulas *(12)*, the median theoretical (thermodynamic) binding ratio of the design allele to the false (second) allele of the 82 probes was 30 (range, 4.2–197). This was clearly sufficient for unequivocal assignment of alleles in the droplets (see the Data Supplement that accompanies the online version of this report at http://www.clinchem.org/content/vol59/issue12). Respective PCR primers were designed to exhibit a melting temperature of 68 °C and a thermodynamic binding efficacy of >95% at 60 °C. Each of the assays was first optimized in a LightCycler480 (Roche Applied Science) by using the ddPCR Supermix for Probes (Bio-Rad Laboratories) and subsequently optimized for the ddPCR with droplet generation *(13, 14)*. Two different annealing temperatures were established to maximize efficiency and differentiation of alleles. Online Supplemental Table 1 lists probes and other characteristics for each of the finally selected SNP assays.

SAMPLE COLLECTION AND PREPARATION

For initial assay establishment and optimization, genomic DNA and cfDNA were extracted from EDTA-anticoagulated blood in Monovettes (Sarstedt) collected from healthy adult volunteers. Within 1 h after collection, plasma was separated from the cells by centrifugation (2500*g* for 10 min at 4 °C, followed by a second centrifugation of the plasma at 4000*g* for 20 min at 4 °C to remove

any cell debris). If not used immediately, plasma and buffy coat were frozen at −20 °C until further processing. DNA from both the plasma (>1 mL) and the harvested buffy coat was extracted with the High Pure viral nucleic acid extraction kit (Roche Applied Science) following manufacturer's instructions.

For the group of stable patients, plasma was taken from the leftover EDTA blood samples sampled in the outpatient clinic for routine blood counts or immunosuppressive drug testing, within 5 h after blood draw. For the sampling during the initial phase after LTx, special tubes for collection of cfDNA were used (Cell-Free DNA™ BCT, Streck). The plasma centrifugation and handling were performed as described above.

PREAMPLIFICATION OF cfDNA

We normally expect about 1000–1500 genome copies in 1 mL of blood. The recovery from 2 mL EDTA plasma therefore, would be expected to be about 4000–6000 copies. If minor allele concentrations of 2% are to be detected in a quantitative manner, the required number of fragments for testing several SNPs can be achieved only with a preamplification. For this purpose, we used the NEBNext Ultra DNA Library Prep Kit (New England Biolabs), because this gave the best efficiency at amounts as low as 5 ng of DNA, which reflects the usual amount of cfDNA, in samples collected as described above. We amplified the ligated cfDNA to 1100 ng on average (SD, 325) with a maximum of 11 PCR cycles using real-time monitoring of library amplification in a LightCycler480 (Roche Applied Sciences). The following PCR conditions were used: 98 °C for 30 s initial denaturation, 8–11 cycles of 98 °C for 10 s, 65 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5min. Reactions contained 1× EvaGreen for real-time monitoring and were stopped immediately, when the amplifications reached their plateau.

DIGITAL DROPLET PCR

The QX100 ddPCR system (Bio-Rad) was used and all reactions were prepared using the ddPCR Supermix for Probes (Bio-Rad). Each reaction contained 30 or 100 ng of the cfDNA library as template, 900 nmol/L of each primer, and 250 nmol/L of each probe. Droplets were generated using the QX100 droplet generator (Bio-Rad) according to the manufacturer's protocols. The cycling conditions were: 95° C for 10 min, 50× (94 ° for 30 s, 59 °/61 ° for 1 min), then 98 °C for 10 min. Droplets were read in the QX100 droplet reader and analyzed using the Quantasoft version 1.3.2.0 (Bio-Rad) software. For the quantification of the minor allele fractional abundance, the embedded "Rare Event Detection" calculation was used, which takes the underlying Poisson distribution into account to calculate the template molecule concentration of either allele. These values are then used to express the minor allele in percentage of the total concentration. The detection and calculation process is exemplified in the online Supplemental Material for 1 SNP assay.

STATISTICS

If not stated otherwise, all data were analyzed using Microsoft Excel™. The calculation of CIs of measured template concentration ratios of ddPCR is embedded in the Quantasoft SW and follows published algorithms *(15)*. For the calculation of dispersion of the preamplified DNA to genomic DNA and cfDNA ratios the SD from the above-mentioned embedded function of each value was used with respective formulas *(16)* to assess the SD of the ratios, for which data were considered correlated.

Results

PERFORMANCE CHARACTERISTICS OF ddPCR SNP ASSAYS

We first investigated whether the limit of quantification of the minor (graft-derived) alleles would be sufficient for the medical needs. To do so, known amounts of genomic DNAs with known genotypes per SNP were mixed at a minor allele concentration of 2%. The intraassay imprecision was determined in a series of 9 repetitions in 1 run to calculate a CV. The CV profiles for 13 of the SNP assays are shown in Fig. 1. It can be seen that even at a 2% minor allele content a CV of <15% (range 4%–14%) was achieved, which is comparable to the theoretically obtainable CV, based on the number of droplets positive for the minor allele (151; SD, 54). The reported CVs were sufficient for the purpose of GcfDNA detection, considering published values *(2)*. The recovery of the spiked 2% minor allele was on average 1.87% (94% of spiked value) over the 13 SNP assays, with an SD of 0.24% (13%).

To assess any potential bias introduced by preamplification *(17)* of the cfDNA, blood samples were taken from 5 healthy volunteers. The cfDNA extracted from approximately 12 mL plasma was split and one-half was subjected to the preamplification procedure. With the use of assays for which the respective donors had a heterozygous genotype, the preamplified material (100 ng) was subjected to ddPCR and compared to the respective native cfDNA (10 ng) and genomic DNA (10 ng). The fractional abundance of the 2 alleles was not found to be significantly different in the preamplified material (Fig. 2) compared with the respective genomic DNA (102% ± 4%) or cfDNA (98% ± 5%). Due to the small difference of only 1 nucleotide between the 2 amplicons per SNP assay, no substantial differences between the fractional abundances occur when moderate numbers of amplification cycles are used.

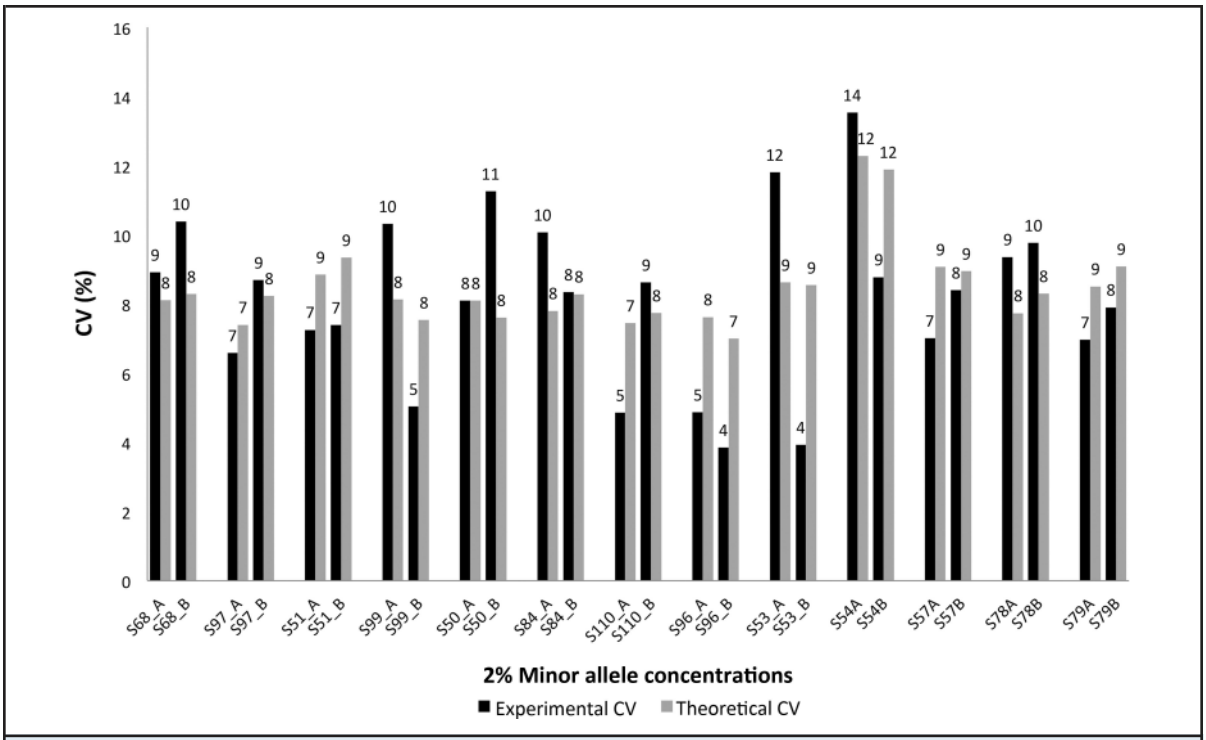

Fig. 1. Bar graph showing CV (%) for 13 assays with 2% minor allele concentration.

For each assay the CVs for 2% of allele A and 2% of allele B are shown (black bars). The intraassay imprecision was obtained in 9 repetitions within the same QX100 run using 100 ng total input DNA. Grey bars show the theoretical CVs as calculated from the number of droplets positive for the minor allele.

Fig. 3 depicts an overview of the procedure employed to determine the graft cfDNA content in the recipients' circulation. To select those SNPs that gave the highest theoretical sensitivity for each recipient, 1 sample was tested for all SNPs in a single (relatively inexpensive) LightCycler480 run using genomic DNA prepared from buffy coat. SNPs heterozygous in the recipient were eliminated from the consecutive ddPCRs. If multiple samples from 1 patient were to be tested, only 1 sample needs to be used for this preselection procedure, which can be done in 1 working day. This approach yielded a mean of 17 (SD, 4) useful informative SNP assays for each of the transplant recipients (n = 33) reported here. The consecutive testing using only the selected SNP assays in the ddPCR for a patient takes about 6 h from receiving the blood sample to the generation of the final result.

GRAFT cfDNA IN CLINICAL SAMPLES

The ddPCRs of the clinical samples were run using 30 ng (LTx samples) or 100 ng (HTx and KTx samples) of the cfDNA library per well, which conforms to about 0.5 and 1.5 copies per droplet, respectively. Fig. 4 shows the results for stable liver, kidney and heart transplant recipients without any signs of rejection. A total number of only 16 different ddPCR SNP assays was used for these samples to achieve the indicated number of informative assays (given below the abscissa for each patient) to determine the GcfDNA percentage. The percentage of graft DNA in the circulation of liver recipients was lower than 10% in all stable LTx patients. The average amount of GcfDNA was 3.5% (range, 1.0%–8.5%) in the LTx group. The mean KTx and HTx GcfDNA contents were lower; 1.2% (0.2%–3.5%) and 0.9% (0.1%–3.4%), respectively.

Very high amounts of graft-derived cfDNA were found in samples collected immediately after reperfusion of the graft in 3 LTx patients analyzed to date (Fig. 5A). During the immediate postengraftment phase, approximately 90% of the cfDNA originated from the graft and decreased with an approximate half-life of 24–48 h. Seven patients were followed early after LTx (see online Supplemental Table 2). In 4 patients who had no severe reported complications or rejection episodes (Fig. 5B), the percentage of GcfDNA was usually <15% from day 10 onward. A total of 12 ddPCR assays were performed for patient LTx1, 16 for LTx3, 7 for LTx4, 18 for LTx6, 5 for LTx7, and 8 for LTx8.

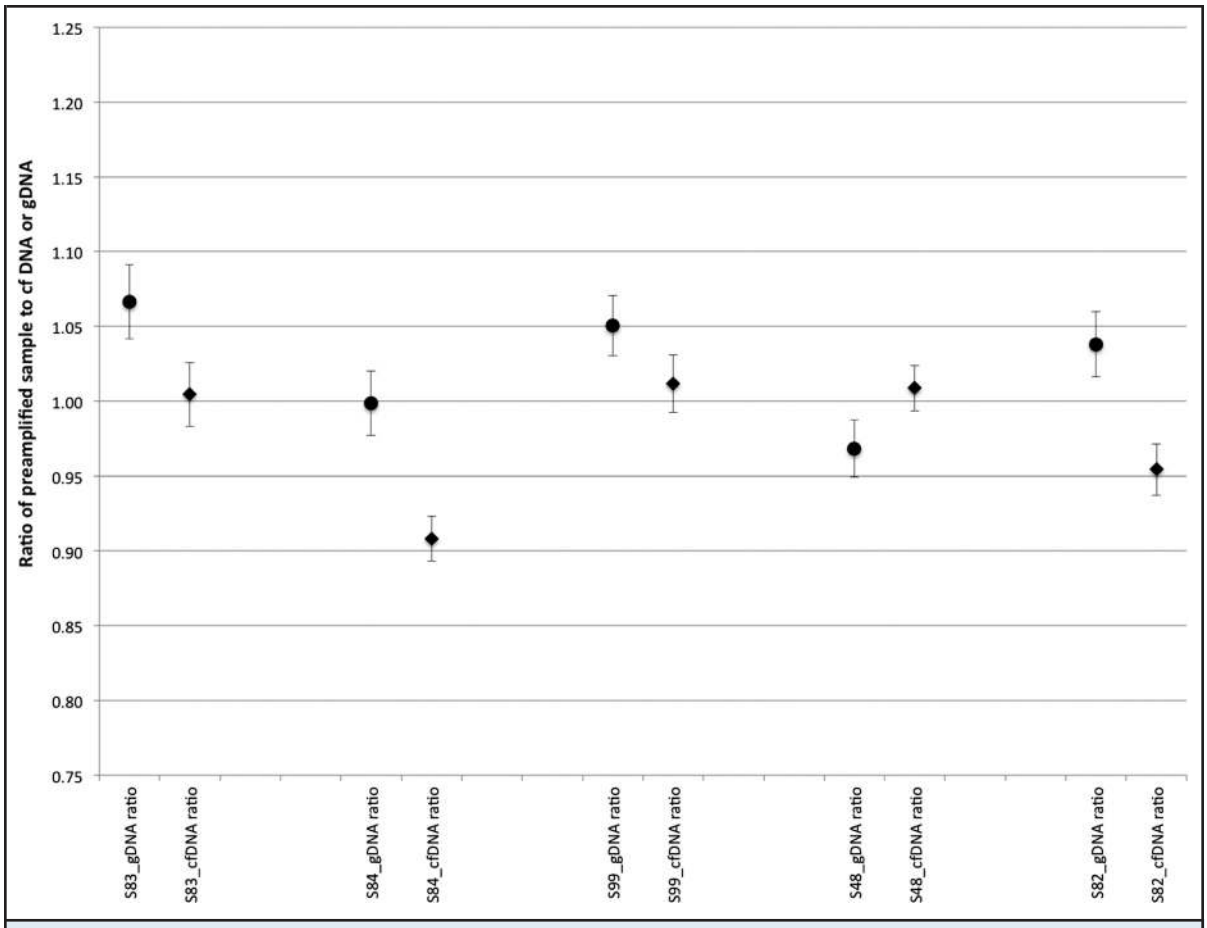

Fig. 2. Results from preamplified cfDNA libraries and direct detection, displayed as ratio of the result in preamplified samples to either genomic DNA or direct cfDNA measurement from the same blood draw.

It can be seen that the preamplification did not introduce a bias into the SNP detection method. Ratios of the copy number ratios with SDs are given.

In Fig. 5 the mean of the informative SNPs for each time point is given.

Results differed in 1 patient (LTx2), who had a rejection episode (proven by biopsy at day 43 after LTx). Five informative ddPCR assays were averaged to obtain the GcfDNA percentage. Fig. 6A displays the time course of the conventionally used liver rejection marker aspartate aminotransferase (AST), together with the percentage of GcfDNA in this patient. After an initial fall of graft cfDNA to a value of approximately 15% on day 7, values gradually increased and showed a marked steady increase up to 60% between day 31 and 38. During this period AST values were variable. With the exception of 1 temporary decrease that occurred after steroid therapy, the GcfDNA content remained high, peaking on day 58 at >60%. In another patient (LTx3, Fig. 6B) an acute rejection episode was diagnosed by biopsy on day 144 after LTx. This rejection coincided with increased graft cfDNA to 19% on day 145 after LTx and further to 55% on day 151. In contrast, 1 patient (LTx1) suffering from an acute cholestasis episode starting at postoperative day 50 showed no increase of graft cfDNA (Fig. 6C). A steady increase in AST, γ-glutamyltransferase (GGT), and bilirubin from day 50 onward peaked around day 160. The cause of the cholestasis was attributable to papillary occlusion and was treated by endoscopic retrograde cholangiopancreatography. During this entire episode, in contrast to the rejection cases, the GcfDNA remained below 10%.

Discussion

In the search for sensitive, specific biomarkers of solid organ graft rejection, the detection of donor (graft) DNA in the recipient's circulation seems promising.

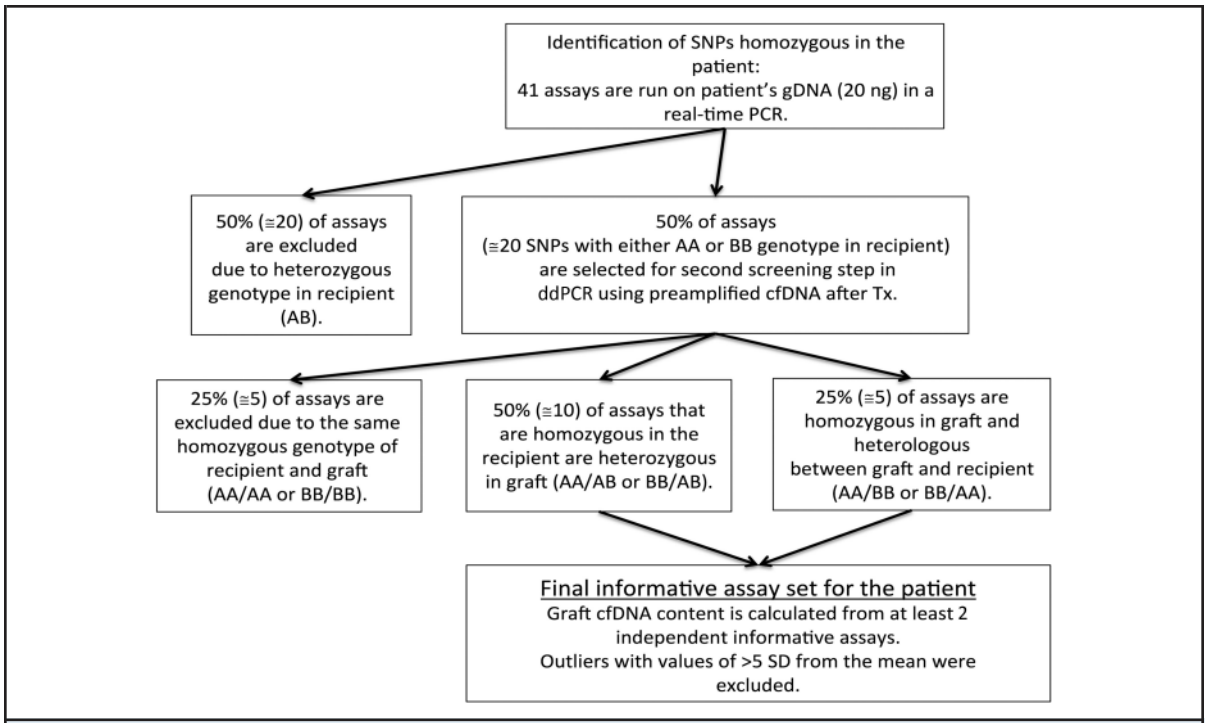

Fig. 3. Scheme of the workflow used to select informative assays and measure GcfDNA.

The first screening step is performed in a real-time PCR using the recipient's genomic DNA (gDNA) as template. In this step all SNP assays for which the recipient has a heterozygous genotype are eliminated, because they cannot be used in the quantification ddPCR. In the next assay selection step, in which preamplified cfDNA is used as the template, the final informative assay set for the individual patient is defined. This step is performed as ddPCR. An informative assay detects an SNP that is homozygous in the recipient and either heterozygous or homozygous in the graft but heterologous between recipient and graft. The latter are the preferred SNP assays for a patient (of which 5 should be found on average), because the relative amount of the GcfDNA will be double compared to the first constellation. The percentages and numbers of assays given for each selection step are calculated for a minor allele frequency of 0.5 and can vary between individual patients.

We have established a combination of assays that allow for the quantification of this potential biomarker at reasonable costs and within 1 working day, which are necessary conditions for the routine clinical use of this biomarker. The availability of devices that are capable of doing automated digital PCR is required for the use of such diagnostic tools in clinical laboratories.

There are 2 major factors limiting long-term outcome in transplantation, namely, irreversible chronic rejection and side effects of standard immunosuppression, such as nephrotoxicity, cardiovascular disease, opportunistic infection, and malignancy. More than 50% of transplanted kidneys from deceased donors fail within 10 years. Conventional KTx tests (e.g., creatinine) are often increased only after substantial tissue damage has already occurred. Such delays in detection can lead to late interventions, with graft damage that can result in chronic rejection. Therefore, better (e.g., molecular) biomarkers are needed *(18)*. Against this background, numerous attempts have been made to develop biomarkers that complement therapeutic drug monitoring to achieve personalized immunosuppression. The main problem with therapeutic drug monitoring alone is that it does not precisely reflect the effects of immunosuppressive drugs on immune cells. Reasons for this include the high between-person variability in sensitivity to immediate and long-term suppression of immune function and the lack of correlation between plasma and intralymphocyte immunosuppressive drug concentrations. Thus, despite careful monitoring, both over- and underimmunosuppression are still common. An exciting new approach for noninvasive early detection of organ rejection is based on the determination of circulating GcfDNA in the recipient. Previous investigations in HTx recipients reported that the percentage of GcfDNA increased substantially during long-term treatment at least 3 months before biopsy-proven rejection *(2)*. These data suggest

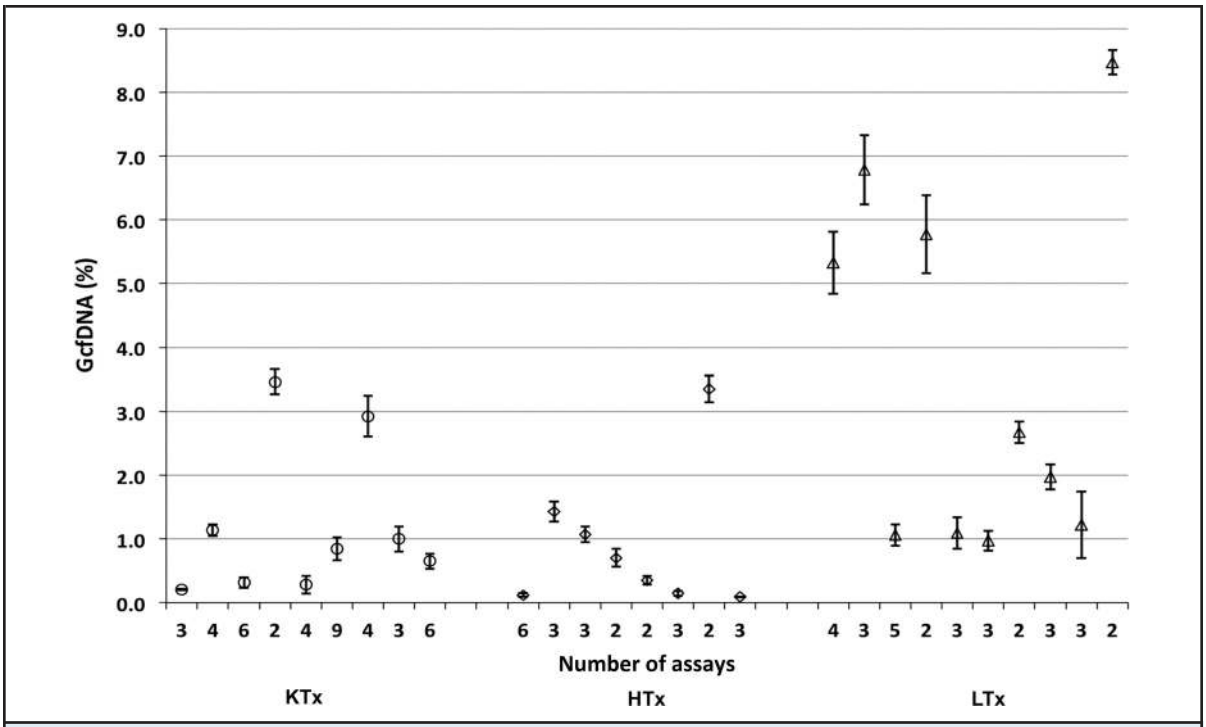

Fig. 4. GcfDNA measured in the circulation of 9 stable KTx, 8 stable HTx, and 10 stable LTx patients (mean and SD are given).

The number of ddPCR SNP assays used for each patient is given below the abscissa.

that it may be possible to use such an assay for early detection of subclinical rejection, which could allow an individual adjustment of immunosuppressive therapy. Conventional tests used in heart transplantation (e.g., echocardiography and cardiac biopsies) are either nonspecific and insufficiently sensitive or invasive, respectively. There are no reliable laboratory biomarkers of HTx rejection. Biomarkers used in KTx may not increase until as much as 50% of organ function has already been lost *(18)*. After LTx, several nonrejection etiologies can increase the values of the commonly used liver function tests used as rejection biomarkers. This new cfDNA approach may be helpful to minimize or even prevent rejection in many if not the majority of organ transplants.

Monitoring GcfDNA has the advantage that it directly interrogates the health of the graft organ and, therefore, could allow for the early detection of cellular or antibody-mediated rejection. Although controlled clinical trials will be needed, it is possible that this methodology could enable effective early intervention to prevent full-blown acute rejection. In our study, we identified 1 LTx case with a complicated course leading to organ rejection 5 weeks after transplantation. A failure of GcfDNA to fall below 15% by 10 days after surgery as well as a very significant rise in the GcfDNA may have been the earliest indicators of rejection. Whether recurrence of hepatitis will also lead to substantial increases of GcfDNA needs to be investigated in larger numbers of patients. Nevertheless even a profound but transient cholestasis did not result in changes in GcfDNA, suggesting the test provides good discrimination between cholestasis and rejection in the initial differential diagnosis.

Overall, the GcfDNA values observed here as well as those reported using other, more complicated methods *(2, 4)* were all well above the method's limit of quantification, and critical changes were above the sensitivity of the ddPCR method used. The relatively higher GcfDNA amounts in stable LTx compared with KTx and HTx that we report here may reflect the larger graft volume and higher regeneration rate usually seen for hepatocytes compared with heart and kidney cells. Although controlled clinical trials are needed, it appears that this method of quantifying GcfDNA in the recipient's circulation has the potential to complement or possibly even replace other approaches used for posttransplant monitoring. The new test may also be useful to monitor immunosuppression minimization, because it might allow the early identification of pa-

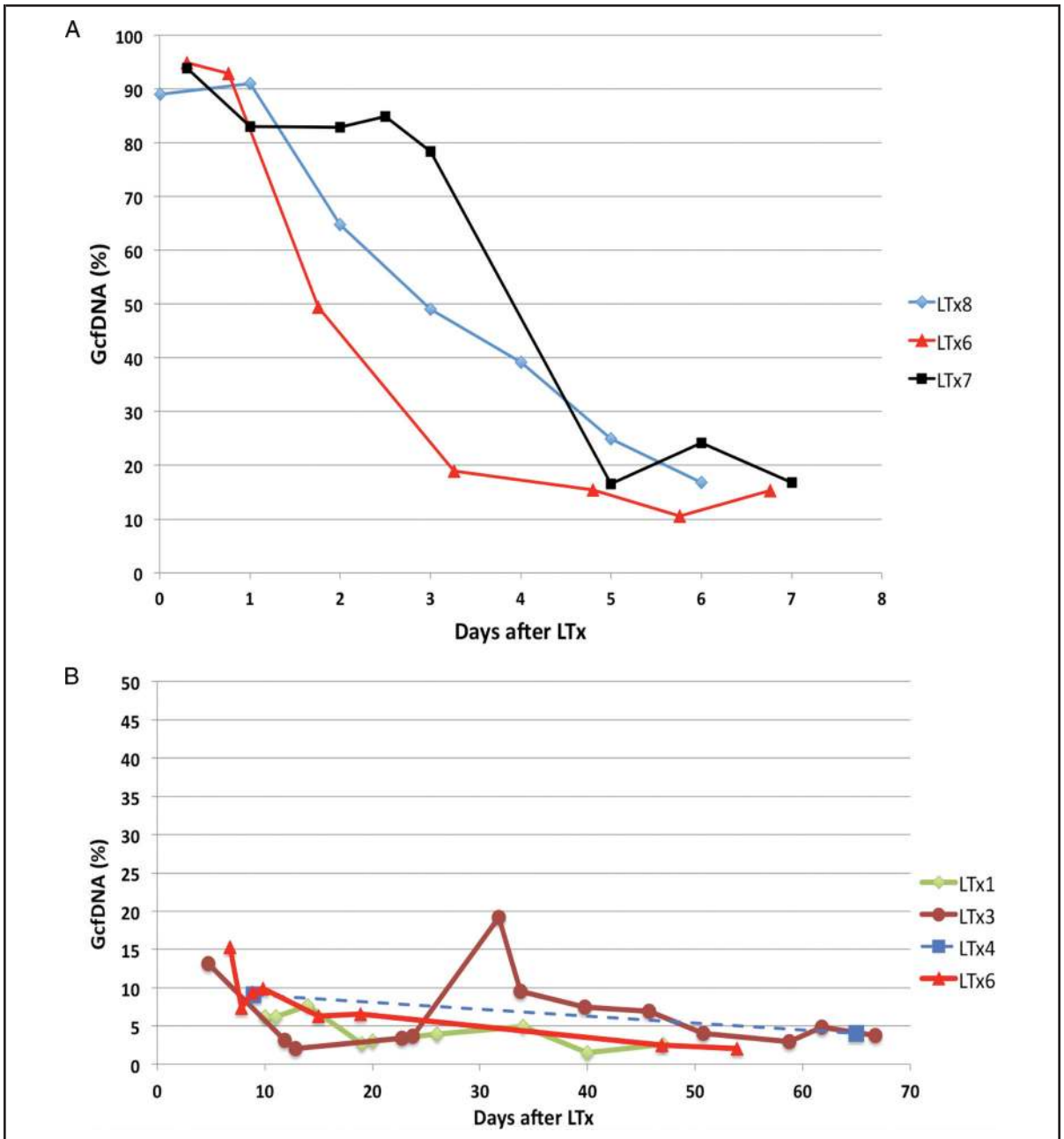

Fig. 5. (A), The variance in the pattern of decrease of GcfDNA seen in 3 LTx patients during the first days after transplantation.

In 1 case there was a rapid decrease, in another there was an initial persistence at higher values, followed by a slightly delayed sharp decrease, whereas the third patient (LTx8) showed a steady, but slow, decrease of GcfDNA. (B), Examples of 4 patients with uncomplicated courses, having GcfDNA ratios usually below 15% after the first week after LTx. The 1 higher single point in patient LTx3 was accompanied by a transient, parallel increase of AST (not depicted).

tients in whom dosage decreases result in acute rejection. A further interesting possible use is the evaluation of the reperfusion phase after transplantation. We observed high GcfDNA concentrations up to approximately 95% in the first few days after surgery. Studies are being done to examine whether these early measurements of GcfDNA, when combined with the dynamics of the decline of these concentrations, reflect

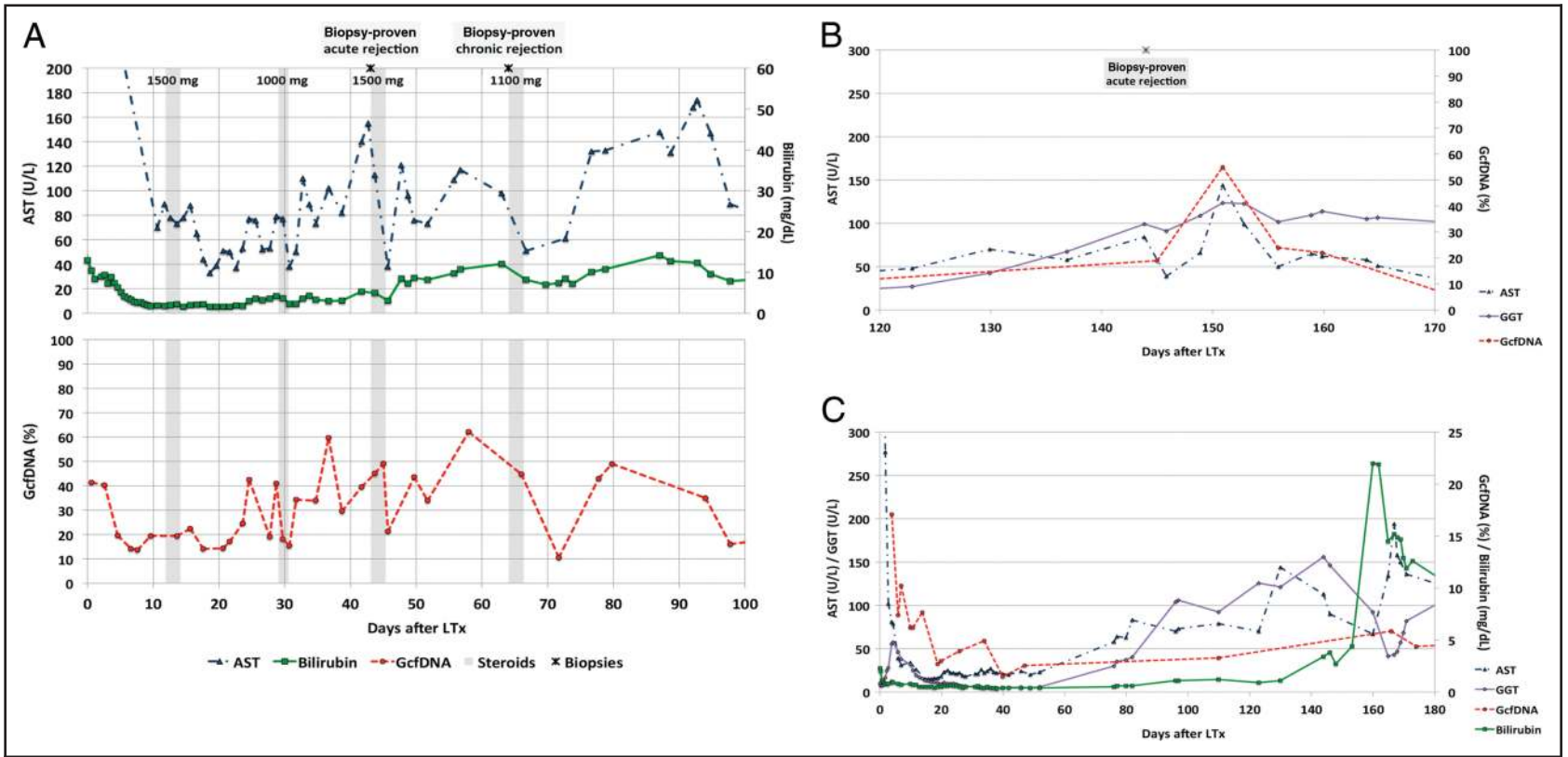

Fig. 6. (A), Time course of measurements in 1 LTx patient with rejection episodes.

GcfDNA content showed a marked and steady increase from day 31 onward, with an initial peak of approximately 60% at day 38. During this period, the conventional biomarker AST was more variable. (Note that the ordinate scale for the GcfDNA is 10-fold of Fig. 4.) (B), Late time course of patient LTx3: a late acute rejection episode was diagnosed at day 144 after surgery. The percentage of GcfDNA was 19% at day 145 and showed a steep increase to 55% at day 151. (C), The late time course of patient LTx1 is shown, including an episode of cholestasis. It is noteworthy that GcfDNA did not rise despite increases in conventional liver function tests.

the severity of ischemia/reperfusion damage. At present there is no definitive measure for the amount of ischemia/reperfusion damage, which is thought to be linked to ultimate long-term graft outcomes.

A novel cost-effective test based on automated ddPCR with adequate same-day turnaround has been developed and used for the first time. Clinical trials need to be done to examine whether this test can be used to improve the chances of long-term graft and recipient survival. Measurement of the GcfDNA in transplant recipients may also provide a helpful tool to achieve personalized immunosuppression and help shift the focus of transplant rejection monitoring from postrejection reaction to earlier intervention and prevention. Effective, noninvasive methods for direct interrogation of transplanted organs may allow safer, more effective therapeutic use of immunosuppressive drugs and thereby reduce the morbidity, mortality, and healthcare costs associated with organ 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.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the author disclosure form. Disclosures and/or potential conflicts of interest:*

Employment or Leadership: J. Beck, Chronix Biomedical; S. Bierau, Chronix Biomedical; S. Balzer, Chronix Biomedical; J. Schmitz, Chronix Biomedical; M. Oellerich, *Clinical Chemistry*, AACC; E. Schütz, Chronix Biomedical.
Consultant or Advisory Role: None declared.
Stock Ownership: J. Beck, Chronix Biomedical; E. Schütz, Chronix Biomedical.
Honoraria: None declared.
Research Funding: Chronix Biomedical and BMBF.
Expert Testimony: None declared.
Patents: J. Beck, US patent application 61/828,553; E. Schütz, US patent application 61/828,553.
Other Remuneration: E. Schütz, Chronix Biomedical.

Role of Sponsor: The funding organizations played a direct role in design of study, review and interpretation of data, and preparation and final approval of manuscript.

Acknowledgments: We thank Howard B. Urnovitz for his revisions and fruitful comments on the manuscript.

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