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# Biology of Blood and Marrow Transplantation

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## Evaluation of Next-Generation Sequencing and Crystal Digital PCR for Chimerism Monitoring of Post-Allogeneic Hematopoietic Stem Cell Transplantation

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### ABSTRACT

Hematopoietic stem cell transplantation (HSCT) is a curative treatment for most hematologic diseases. To evaluate the level of donor engraftment, chimerism must be carefully monitored after HSCT. Short tandem repeats, quantitative PCR (qPCR), and, more recently, digital PCR (dPCR) are widely used to determine the proportions of donor and recipient cells after HSCT. The screening and quantification of chimerism have been evaluated by 2 new methods: a ready-to-use next-generation sequencing (NGS)-based method using the Devyser ChimerismNGS kit and an original combination of the Stilla crystal digital PCR (cdPCR) platform with 3-color multiplexing capacity using GenDX KMRtrack reagents. The genotyping of 4 HSCT pairs by cdPCR using 11 triplex mixes of the GenDX KMRtype kit was consistent at 98.8% with qPCR. Informative samples ( $n = 20$ ) from 6 donor-recipient pairs and 1 external proficiency test demonstrated the reliability of the results (0.1% to 50%) for the 2 methods. The methods are also highly sensitive (0.1%) and accurate. The chimerism values of the 2 methods are correlated and concordant with those of the reference methods. In addition, the ADVYSER software (Devyser) is user-friendly and well adapted to chimerism monitoring. In conclusion, these 2 innovative methods are easy to perform and user-friendly in all molecular, hematology, and immunogenetic laboratories and allow the genotyping and monitoring of chimerism with high performance and sensitivity.

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### INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is a curative treatment for most hematologic diseases. To evaluate the level of donor engraftment, chimerism must be carefully monitored after HSCT. Chimerism monitoring is very important in the engraftment phase to detect graft failure, as well as post-HSCT to detect the risk of disease relapse in malignant disease [1,2]. Currently, 2 methods, short tandem repeat (STR) PCR and quantitative PCR (qPCR), are routinely performed in laboratories. However, their range of analysis—5% to 80% for STR PCR and 0.1% to 30% for qPCR—and the need for 2 technical

steps—genotyping of genetic markers of the donor-recipient pair and then quantification of chimerism using informative recipient markers—limit their applications. More recently, we and others have shown that droplet digital PCR (ddPCR) using chimerism quantification kits, specific or not for this ddPCR method (CE-IVD marked or not) is an effective alternative. The quantification range of this method is at least 0.1% to 90% [3–5].

Among the advantages of ddPCR over qPCR are that ddPCR enables the multiplexed absolute quantification of the target and endogenous genes in the same assay and does not require a standard curve. Furthermore, ddPCR detects the target at the endpoint of amplification, resulting in a lower sensitivity of PCR inhibitors. However, the genotyping step is difficult to integrate and thus must be performed using a qPCR platform. Stilla Technologies (Villejuif, France) has developed the first dPCR system with 3-color multiplexing capacity. The Naica

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system performs dPCR using a hybrid approach, termed crystal digital PCR (cdPCR), combining a dPCR chamber, which relies on 2D arrays of microchambers to partition the sample, and the use of partitions as implemented in ddPCR [6]. This multiplexing cdPCR approach can decrease the number of wells used to identify informative markers for HSCT pairs.

Next-generation sequencing (NGS) technologies have revolutionised the field of genomics, and their applications have been extended to different fields, including clinical diagnostics and forensic science [7,8]. In this context, several custom NGS chimerism panels using single-nucleotide polymorphism (SNP) markers have been applied to chimerism and have shown close concordance with historical results from STR analysis. Their sensitivity has reached 0.5% to 1%, which exceeds that of STR analysis [9,10]. These panels are characterized by many selected SNPs, which have a heterozygosity frequency of approximately 0.5 for the European population to obtain numerous informative markers for each HSCT pair and are located in different regions of each chromosome, thus avoiding false-negative results caused by chromosomal deletions during relapses of a few malignancies [11]. Moreover, the genotyping of genetic markers of donor-recipient pairs and quantification of chimerism are performed in the same protocol, which takes between 36 and 48 hours. Today, several commercially available kits offer an NGS method for chimerism as a complete workflow solution for laboratories, combining a reliable testing process with purpose-designed analytical software.

In the present study, we evaluated 2 new chimerism quantification methods, Crystal Digital PCR (Stilla Technologies, Villejuif, France) using KMR kits (GenDX, Utrecht, The Netherlands) and NGS using the Devyser Chimerism kit (Devyser AB, Hägersten, Sweden). We first evaluated the capacity of these 2 methods to identify informative markers for HSCT pairs for use in chimerism monitoring. We then evaluated whether these 2 methods are sufficiently accurate and sensitive for monitoring chimerism by quantitative determination of mixed chimerism using an artificial DNA mixture and DNA from HSCT recipients.

## METHODS

### Biological Samples

Artificial chimeric samples were prepared from the donor samples of 2 healthy subjects (1 male and 1 female). To study the linearity of measurement, we prepared DNA dilutions of the male and female samples at concentrations of 50%, 10%, 1%, 0.5%, and 0.1%.

A total of 43 samples from 13 patients with malignant hematologic diseases treated with allogeneic HSCT were included in this study (Table 1). The underlying diseases were immunodeficiency (ID) in 3 patients, thalassemia (Th) in 1 patient, acute myelogenous leukemia (AML) in 3, acute lymphoblastic leukemia (ALL) in 5, and myelofibrosis (MF) in 1. Two samples were collected at the time of acute leukemia relapse. The chimerism of each sample had been previously quantified by STR analysis using the AmpFLSTR Identifier PCR Amplification Kit (Applied Biosystems, Warrington, UK) and/or qPCR using the JETA Molecular Kit (JETA Molecular, Utrecht, The Netherlands).

For comparisons between different techniques, 17 samples (15 whole blood, 1 CD3 isolation, and 1 bone marrow) were obtained from 6 pediatric patients and 3 samples (0.1%, 2%, and 10% expected chimerism) were obtained from an external quality control (Société Francophone Histocompatibilité et Immunogénétique, External Proficiency Testing). These samples were selected for their informativeness concerning the Y chromosome. Chimerism was previously evaluated by ddPCR (Bio-Rad, Hercules, CA) using a specific probe for the Y chromosome (KMR54; GenDX) and a nonpolymorphic endogenous gene (*RPP30*) [5].

For the other samples, 26 samples (20 whole blood, 3 bone marrow, 2 CD3 isolation, and 1 CD33 isolation) were obtained from 7 patients (4 adults and 3 pediatric patients). Sixteen chimerism analyses from 11 STR samples and 5 qPCR samples were performed by NGS, and 10 chimerism analyses from 2 STR samples and 8 qPCR samples were performed by cdPCR.

All the donors and patients provided written informed consent, after which the clinical data were collected from each patient. The signed informed consent forms were recorded as a part of the patient's clinical record.

### DNA Isolation Methods

The genomic DNA of the donors (peripheral whole blood) and recipients (whole blood, CD3 and CD33 isolation, and bone marrow) were isolated using the QuickGene 610 L nucleic acid isolation system (Kurabo Industries, Osaka, Japan) with a DNA blood kit. The DNA concentration and purity were checked by absorbance measurement using an ND-One spectrophotometer (Nanodrop Technologies, Wilmington, DE). All the DNA used in the study had absorbance ratios of A260/A280 >1.8 and A260/A230 >1.8. The DNA samples were stored at  $-40^{\circ}\text{C}$  for <48 months before use in experiments.

### Chimerism Analysis by NGS

The principles and technical characteristics of NGS are described in Table 2. The chimerism level was quantified using the Devyser Chimerism kKit following the manufacturer's instructions. This kit is based on multiplex PCR using a range of 24 highly informative markers distributed over 17 chromosomes suitable for the screening of a recipient-donor pair and monitoring of chimerism status followed by specific Illumina NGS library preparation and sequencing. The resulting sequences are analyzed using ADVYSER for Chimerism version 1.3. This software performs identification of donor- and recipient-specific markers, calculation of the chimerism percentage, automatic generation of trending charts to assess monitoring results, and parallel trending of various cell populations.

Genotyping is determined by the % variant allele frequency (VAF) calculation for each marker using the number of reads classified as "reference" (Ref) and "alternative" (Alt) of the reference sequence hg19 (human genome 19; Genome Reference Consortium). The %VAF is calculated as follows:  $\text{Ref reads} / (\text{Ref reads} + \text{Alt reads})$ . Pretransplantation samples are expected to have a %VAF close to 0% (-/-), 50% (+/-), or 100% (+/+). A marker is informative if the recipient and donor are homozygous for opposite genotypes or if the recipient is heterozygous and the donor is homozygous (+/+ versus -/- or -/- versus +/- and +/- versus +/- or +/-, respectively). All the informative markers are selected automatically, but the user can deselect them manually. Three warnings should be considered for the selection of the markers: "low coverage," when the minimum recommended coverage of 100 reads/marker is not met; "unexpected VAF," when the %VAF is between 1% and 40% or between 60% and 99% (due to import mix-up; ie, a post-transplantation sample is imported instead of a pretransplant sample), with sample impurity (ie, as detected in saliva swabs) or if the patient has had previous transplantations; and "background noise," when the %VAF is between 0.1% and 1% or between 99% and 99.9% (due to a nonoptimal run, index hopping, or a carryover event). For monitoring, there are 2 quality criteria: coverage and noise. For each of these, there are 2 levels of warning: "yellow" and "red." The optimal coverage depends on the % chimerism. A coverage >10,000 reads is required to call chimerism at .1% with high precision and sensitivity. If the detected % chimerism is higher (>1%), the a coverage of >1,000 reads/marker is sufficient to determine the % chimerism with high precision and sensitivity.

### Chimerism Analysis by cdPCR

The principle and technical characteristics of cdPCR are described in Table 2. Chimerism was quantified using the Naica cdPCR platform (Stilla Technologies) with a 3-color target multiplexing capability (detection wavelengths: 495 to 520 nm, 560 to 610 nm, and 655 to 720 nm). Pretransplantation genotyping and chimerism quantification were performed using GenDX KMRtype and KMRtrack (KMR54: Y-linked probe) CE-IVD qPCR markers, respectively. For 1 sample, we used 10  $\mu\text{L}$  of Perfecta 2x UNG, 2.50  $\mu\text{L}$  of fluorescein (1  $\mu\text{M}$ ), 6.75  $\mu\text{L}$  of 5x GenDX KMR54 FAM, 1.35  $\mu\text{L}$  of 20x RPP30 gene control HEX, and 6  $\mu\text{L}$  of 25 ng/ $\mu\text{L}$  DNA (150 ng). The PCR mix was pipetted into each of 4 inlet ports of Sapphire chips. Three Sapphire chips were positioned into the Naica geode, which was programmed to perform the sample partitioning step, followed by the PCR thermal cycling program: 95  $^{\circ}\text{C}$  for 10 minutes, followed by 45 cycles of 95  $^{\circ}\text{C}$  for 10 seconds and 60  $^{\circ}\text{C}$  for 15 seconds. Image acquisition was performed using the Naica Prism3 reader with the following exposure times: blue signal, 100 ms; green signal, 50 ms; red signal, 50 ms. cdPCR analysis was carried out in 2 h and 30 minutes. The data were analyzed using Crystal Miner software, which allows visual and intuitive quality control of the results by jointly displaying images of the drop crystals and corresponding point plots. These quality controls will enable checking of the uniform distribution of positive partitions across the drop crystals and distinguishing true positives from false positives caused by dust particles in the droplets. The droplet number must be >18,000. Crystal Miner version 2.1.6 automatically calculates and sets an optimal threshold for discriminating between positive and negative drops. This threshold can be modified manually. Based on the chosen fluorescence threshold, the software automatically calculates the concentration of target nucleic acid for

**Table 1**

Data for the Allogeneic HSCT Recipients Included in the Study and Their Clinical and Chimerism (% Recipient) Follow-Up

Patient ID	Sample	Indication	Days after Transplantation	Follow-Up	ddPCR, %	cdPCR, %	NGS, %	qPCR, %	STR, %
1	WB	AML	20	Relapse	0.6	0.7	0.8	0.7	95
	WB		1698		96	90	96		
2	WB	ALL	30		2	2	2	2	
3	WB	ALL	90		0.3	0.35	0.3	0.4	
	WB		140		0.1	0.1	0.1	0.1	
	BM		168		0.3	0.3	0.2	0.3	
4	WB	Immunodeficiency	15		60	55	55		55
	WB		30		50	50	50		50
	WB		367		15	17.5	17	15	
	WB		1260		12	14	14	12	
5	WB	Immunodeficiency	1294		67	64	67.2		60
	WB		3128		60	56	60.7		60
	CD3		3128		54	57.5	56		50
	WB		3288		63	60.5	63	60	
6	WB	ALL	231		0.4	0.3	0.4	0.4	
	WB		399		0.8	0.6	0.6	0.8	
	WB		521		0.4	0.4	0.4	0.4	
7	BM	ALL	90				45		44
	WB		147				15		16
	WB		153				12	10.5	
	CD3		153				0.9	0.5	
	WB		175		Relapse		29		20
8	WB	AML	421				<0.1	<0.1	
	CD33		421				0.3	0.3	
	BM		421				0.3	0.3	
9	WB	AML	70				2.7		<5
	CD3		70				14.2		14
	WB		189				<0.1		<5
	BM		189				1.5		<5
	WB		224				0.1		<5
10	WB	Myelofibrosis	28				0.1		<5
	WB		35				0.1		<5
	WB		121				0.1		<5
11	WB	Thalassemia	381		50	53			50
	WB		520		55	54			55
12	WB	Immunodeficiency	182		6	5		6	
	WB		276		5	5		4	
	WB		367		5	5		5	
13	WB	ALL	28		0.1	0.1		0.1	
	WB		91		0.1	0.1		0.1	
	WB		178		<0.1	<0.1		<0.1	
	WB		265		0.1	0.1		0.1	
	WB		370		<0.1	<0.1		<0.1	

WB, whole blood; BM, bone marrow; AML, acute myelogenous leukemia; ALL, acute lymphoblastic leukemia.

each fluorescence, along with 95% confidence intervals. The results are displayed in tabular or graphic form. The GenDX KMRtype kit comprises 39 individual markers with 13 multiplexing PCR mixes, using 3 probes with mixes carrying FAM, ORG560, and RED610 as dyes. The probes carrying the FAM fluorophore were detected in the 495- to 520-nm wavelength window, probes carrying ORG560 were detected in the 560- to 610-nm wavelength window, and probes carrying RED610 were detected in the 655- to 720-nm wavelength window. In this study, we evaluated 21 markers in 11 triplex mixes (mix 1 and mixes 4 to 13), representing >90% of the informative probes usually used in our laboratory.

For quantification, the informative “recipient” or “donor” marker has a FAM fluorescence, and the nonpolymorphic gene (*RPP30*) has a HEX fluorescence. Quantification of donor or recipient chimerism is obtained by the ratio of the number of droplets to the informative target/nonpolymorphic target ( $\times 2$ ). The limit of blank and limit of detection were assessed using 3 series, including 30 female samples, and recording the number of false-positive events for the Y-probe (KMR 54).

### Statistical Analysis

The square of the Pearson correlation coefficient ( $R^2$ ,  $P$  value) was computed to determine the replicability of the model (cdPCR, NGS, and ddPCR). A Bland-Altman plot was used to visualize the grade of concordance between the results of chimerism obtained by cdPCR and NGS methods.

## RESULTS

### Pretransplantation Genotyping Using cdPCR

Of 168 markers tested (4 donor-recipient pairs) from 21 markers of 11 triplex mixes, 166 (98.8%) were consistent with those previously performed with qPCR. Only 2 markers in different samples were negative, whereas the expected results were positive: the KMR33 and KMR44 for both dyes was FAM (Table 3).

**Table 2**

Principles and Technical Characteristics of cdPCR and NGS

Parameter		cdPCR	NGS
Principles		Selection of donor-recipient informational markers	Multiplex PCR amplification of 24 informative markers (ins/del –75 bp), distributed on 17 autosomal chromosomes >1 tube per sample
		DNA and marker compartmentalisation in thousands of microreactors	Preparation of an Illumina-compatible library: amplicon type >1 tube per sample
		Amplification of microreactors	Paired-end sequencing 2 × 75 bp
		Absolute quantification by digital reading (0 or 1)	Automatic data analysis (genotyping and monitoring) and longitudinal follow-up of patient records
		Chimerism calculation by the informative marker/reference ratio	
Genotyping		Independent experience in addition to quantification	Same protocol as for quantification
	Duration	Hands-on time: 00:20 Instrument times: 2:30 (PCR) - 00:30 (reading)	Hands-on time: 00:20 (amplification) to 1:00 (library prep); instrument times: 1:45 (amplification) to 1:55 (library prep) ~10 h to 28 h (MiSeq)
	Number of markers	21 (in triplex)	24
	DNA quantity	10 ng per well	60 ng
	Number of points	Run of 12 wells per sample	One well per sample
	Screening expertise	Expertise in the choice of 1 or 2 informative donor and recipient markers	No chimerism expertise required; modifiable selection of information markers by the software
Monitoring	Duration	Hands-on time: 00:20 Instrument times: 2:30 (PCR) - 00:30 (reading)	Hands-on time: 00:20 (amplification) to 1:00 (library prep); instrument times: 1:45 (amplification) to 1:55 (library prep) ~10 h to 28 h (MiSeq)
	DNA quantity	150 ng	60 ng
	Maximum number of patients	12	Up to 96 (depending on the flow cell and index number)
Interpretation	Expertise	+	–

**Analytical Performance of cdPCR Chimerism**

The limit of detection was achieved by testing the same female DNA 30 times with the KMR54. The value of the detection limit was 0.663 copies/ $\mu$ L for 150 ng [12]. Quantification of chimerism by cdPCR was reliable over a wide range of measurements (from 0.1% to 50%; 3 points%). The correlation coefficient was  $R^2 = 0.999$  ( $P < 0.001$ ) for the whole range (Figure 1A) and  $R^2 = 0.995$  ( $P < 0.001$ ) for low values (from 0.1% to 1%) (Figure 1B). The coefficients of variation (CVs) of reproducibility were < 20% for all recipient chimerism percentages (Figure 2). The sensitivity of this method reached 0.1%.

**Pretransplantation Genotyping Using NGS**

The screening of recipient and donor samples showed informative markers for all 6 HSCT recipients. Between 3 and 11 informative polymorphisms were automatically identified for each donor-recipient pair, producing the expected percentage of chimerism. If the laboratory selection rules are applied—ie, the SNP was present exclusively in the donor or recipient in the homozygous or heterozygous state (+/+ versus +/- or +/- versus -/-)—similar results to those from automatic rules were obtained (Table 4). Rare cases were identified with the “Background noise” warning, and only 1 case had the “Unexpected VAF” warning (external proficiency testing sample), with no impact on the result of chimerism quantification.

**Analytical Performance of NGS Chimerism**

The quantification of chimerism by NGS was reliable over a wide range of measurements (from 0.1% to 50%, 3 points%). The correlation coefficient was  $R^2 = 0.999$  ( $P < 0.001$ ) for the whole range (Figure 1A) and  $R^2 = 0.974$  ( $P < 0.001$ ) for low values (from 0.1% to 1%) (Figure 1B). The CVs of reproducibility were < 20% for all chimerism percentages (Figure 2). The sensitivity of this method was 0.1%, with a coverage of at least > 10,000 reads for each marker. In our assays, no nonconforming quality criteria were observed.

**Comparison of Chimerism Quantification**

Chimerism quantification by cdPCR and NGS methods were correlated more strongly with the ddPCR technique (Figure 3A). The correlation coefficient was  $R^2 = 0.995$  ( $P < 0.001$ ) for the cdPCR method and  $R^2 = .997$  ( $P < 0.001$ ) for the NGS method. The Bland-Altman plot confirmed a concordance of the results for % chimerism in cdPCR and NGS with ddPCR (Figure 4). Chimerism quantification by the NGS (Figure 3B) and cdPCR (Figure 3C) methods are correlated more strongly with the qPCR and STR techniques.

**DISCUSSION**

This study evaluated 2 new methods, an original combination of cdPCR using GenDX reagents and a ready-to-use NGS-based method using the Devyser Chimerism for NGS kit to screen and quantify the chimerism. Interestingly, cdPCR is a system with 3-color detection capability that provides multiplexing power. We tested 11 triplex mixes of the GenDX KMRtype kit to discriminate informative donor-recipient pairs by qPCR. The 3 dyes of this kit are not completely adapted to their use by cdPCR, because the ORG560 and RED610 fluorophores have wavelengths located at the end of the second signal of the Naica platform. Manual compensation was performed to obtain a more specific signal for these 2 fluorophores. Thus, no assignment error, negative or positive, was found for these 2 fluorophores in the other cases. Inexplicably, the only 2 polymorphism identification errors involved 2 different probes associated with the FAM fluorophore. These probes had been positive and negative at least once without error of identification for the other genotyping. The design of these probes might not be completely suitable for cdPCR. More genotyping is warranted to determine whether these errors are reproducible.

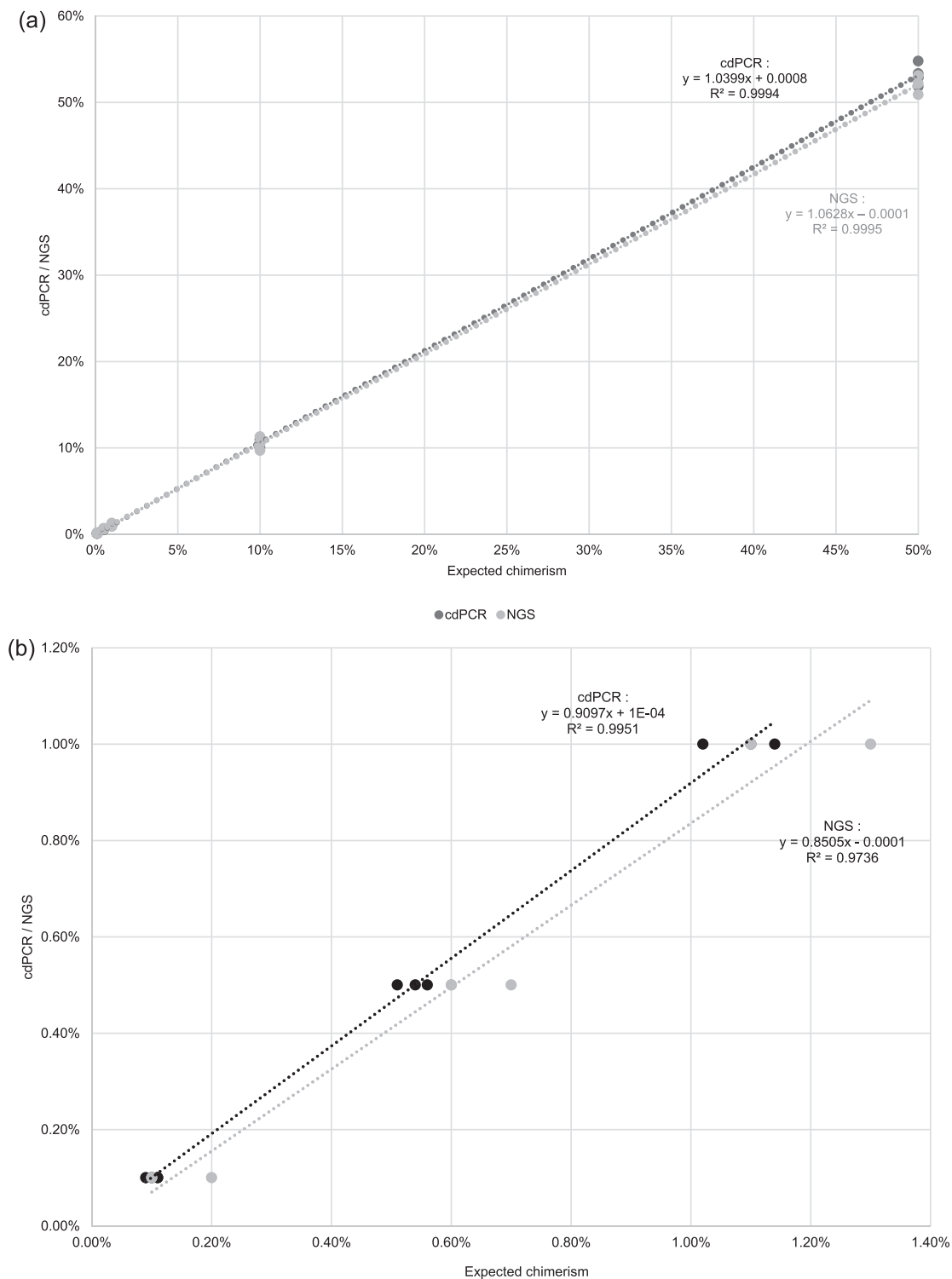
Informative markers of genotyping by NGS of each donor-recipient pair were automatically identified by the ADVYSER software. Each pair had at least 3 donor or recipient

**Table 3**

Comparison of HSCT Genotyping by cdPCR and by qPCR using the GenDX KMRtype Kit

KMR		Recipient 1		Donor 1		Recipient 2		Donor 2		Recipient 3		Donor 3		Recipient 4		Donor 4	
		qPCR	cdPCR	qPCR	cdPCR	qPCR	cdPCR	qPCR	cdPCR	qPCR	cdPCR	qPCR	cdPCR	qPCR	cdPCR	qPCR	cdPCR
FAM	KMR004	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	KMR009	+	+	+	+	-	-	-	-	-	-	-	-	+	+	+	+
	KMR014	+	+	+	+	-	-	+	+	-	-	-	-	+	+	-	-
	KMR033	+	+	-	-	+	+	+	+	-	-	+	+	-	-	+	-
	KMR044	+	-*	+	+	-	-	-	-	+	+	-	-	+	+	+	+
	KMR050	+	+	+	+	+	+	-	-	-	-	-	-	+	+	+	+
	KMR055	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ORG560	KMR013	-	-	+	+	+	+	-	-	-	-	-	-	+	+	-	-
	KMR016	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-
	KMR017	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+	+
	KMR019	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	KMR034	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	KMR051	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
	KMR052	-	-	-	-	+	+	-	-	+	+	+	+	-	-	+	+
RED610	KMR011	-	-	+	+	+	+	+	+	-	-	-	-	+	+	-	-
	KMR020	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-
	KMR029	+	+	+	+	-	-	+	+	+	+	+	+	+	+	+	+
	KMR030	+	+	-	-	+	+	+	+	+	+	+	+	-	-	-	-
	KMR031	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+
	KMR053	-	-	-	-	+	+	-	-	+	+	+	+	+	+	+	+
	KMR054	+	+	-	-	+	+	-	-	-	-	+	+	-	-	+	+

\* Discordance.

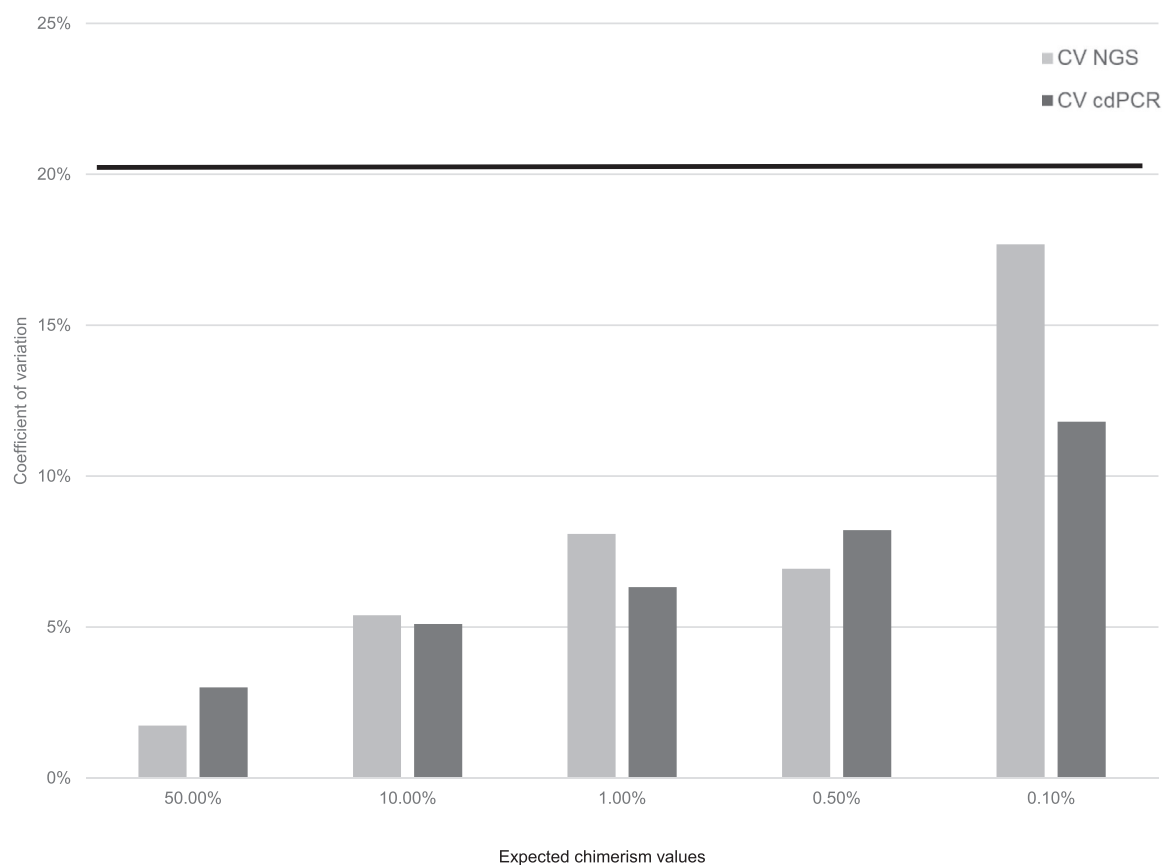


**Figure 1.** Analytical performance of cdPCR and NGS against reference values. A series of artificial chimerism mixtures, ranging between 0.05% and 50%, were quantified by cdPCR and NGS and plotted against their reference values. (A) Whole range. (B) Low chimerism, <1%.

informative markers identified. Manual changes, considering homozygous donor or recipient polymorphisms only as informative, did not change the chimerism quantification results, supporting the automatic selection of informative polymorphisms. Interestingly, the Devyser panel comprises markers with a low bias from ethnic parameters, allowing robust discrimination when analyzing HLA-identical siblings.

Our study shows that these 2 methods have very similar analytical capacities for chimerism quantification. The technical approaches have a similar sensitivity (ie, 0.1%), a range of analysis from 50% to 0.1%, and reproducibility with a CV <20%. In addition, they allow the accurate identification of quantitative differences in the presence of low levels of chimerism. The quantitative results show close concordance with the results from ddPCR, validating these 2 methods for chimerism monitoring.





**Figure 2.** Reproducibility of the cdPCR and NGS methods. Each % chimerism was tested on three different experiments to calculate the CV of each method.

Thus, quantification of chimerism by cdPCR is a sensitive and accurate method (ie, no requirement for standard curves, efficiency controls, and multiple replicates as for qPCR) for monitoring HSCT recipients. All dPCR steps are performed in a single and ready-to-use consumable. Consequently, the Naica system is a quick and easy solution. Moreover, this method is adapted to clinical emergencies (results in <4

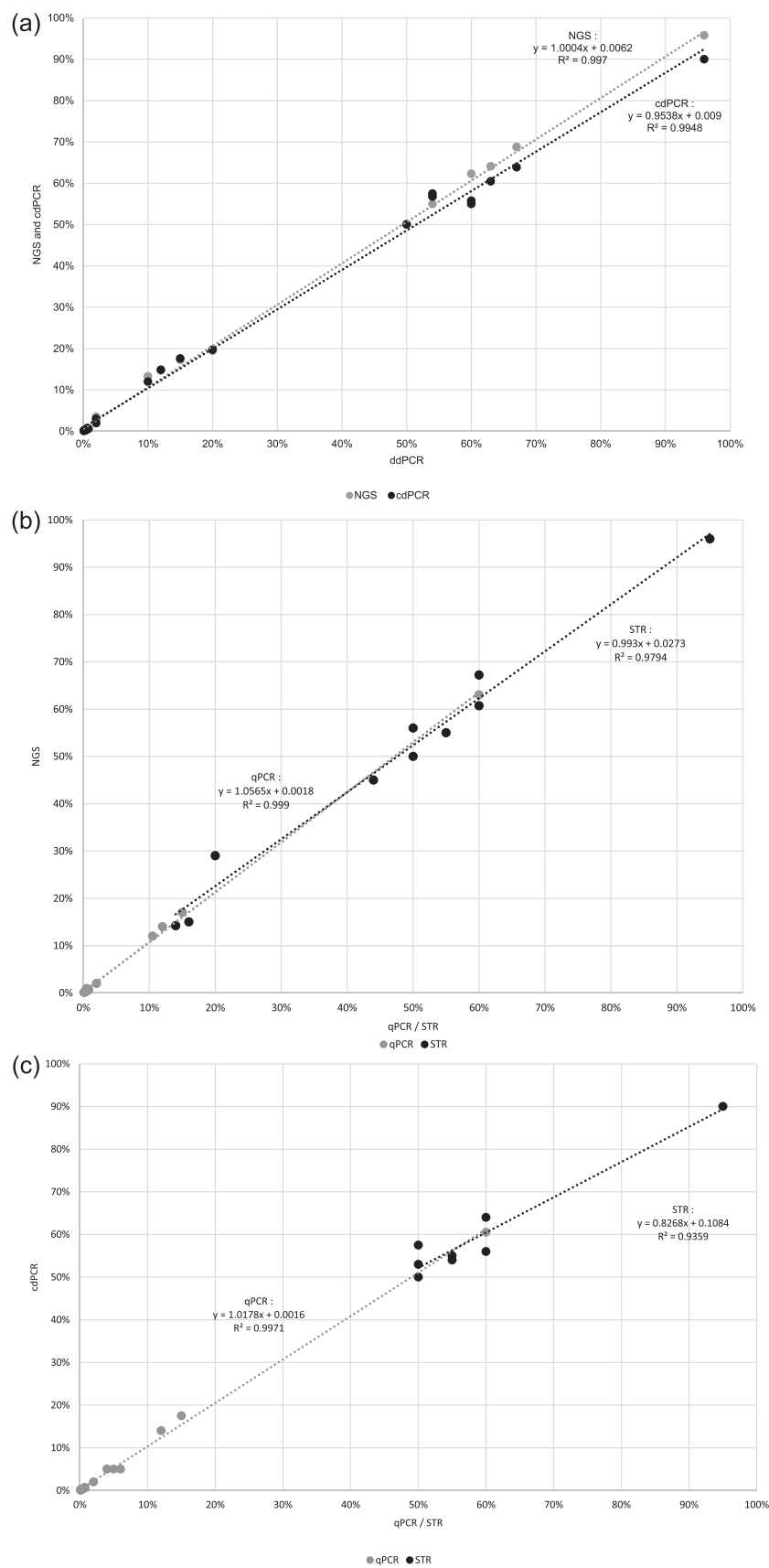
hours). Interestingly, the droplets in the chip can be stored for up to 8 days at room temperature before reading. This makes it possible to perform runs during the night with a reading in the morning. The only technical limitation identified was the phenomenon of electrocoalescence, leading to the fusion of droplets in the chip. The use of an antistatic spray at each run controlled this effect.

**Table 4**

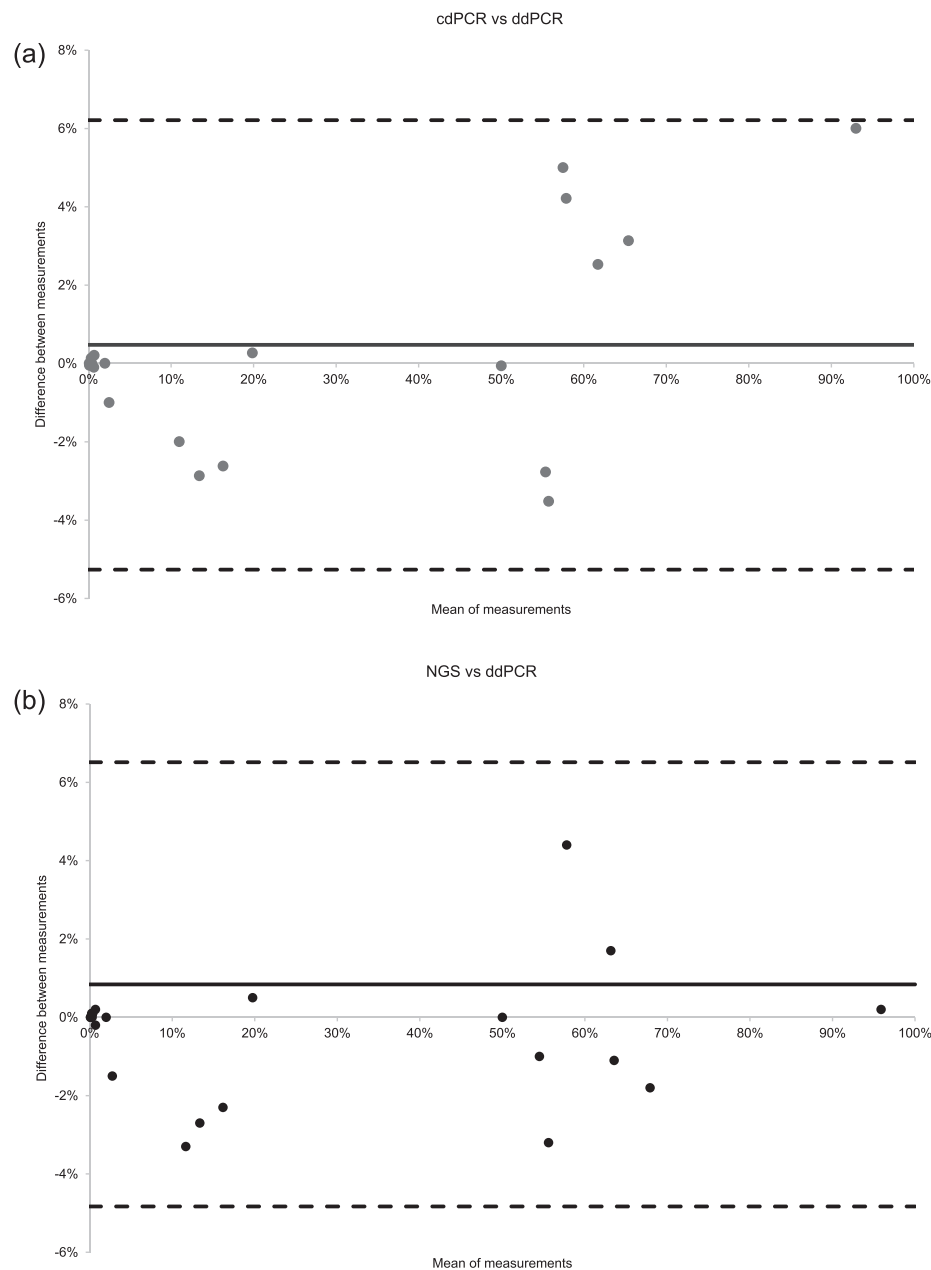
Comparison of the % Chimerism Results from NGS According to the Automatic and Manual Selection Criteria of the Informative Markers

Patient	ddPCR Results, %	Devyser Rules		Laboratory Rules	
		Informative Marker	Result, %	Informative Marker	Result, %
1	0.60	10	0.80	8	0.80
1	96.00	10	95.80	8	96.30
2	2.00	10	2.00	6	1.70
3	0.10	3	0.10	3	0.10
3	0.30	3	0.20	3	0.20
3	0.30	3	0.30	3	0.30
4	50.00	8	50.00	5	50.00
4	60.00	8	55.60	5	55.70
4	15.00	8	17.30	5	17.10
4	12.00	8	14.70	5	14.70
5	67.00	5	68.80	4	67.20
5	60.00	5	62.30	4	60.70
5	54.00	5	57.20	4	56.00
5	63.00	5	64.10	4	62.90
6	0.40	9	0.40	7	0.40
6	0.80	9	0.60	7	0.60
6	0.40	9	0.30	7	0.40





**Figure 3.** (A) Correlation of the % chimerism results between cdPCR and ddPCR and between NGS and ddPCR using regression curve analysis. (B) Correlation of the % chimerism results between NGS and qPCR/STR using regression curve analysis. (C) Correlation of the % chimerism results between cdPCR and qPCR/STR using regression curve analysis.



**Figure 4.** Concordance of the % chimerism results between cdPCR and ddPCR (A) and between NGS and ddPCR (B) using the Bland-Altman plot. The Bland-Altman plot includes the mean value (solid line) and 95% CI (dashed lines). A comparison of results between cdPCR and ddPCR and between NGS and ddPCR shows that all data points were within the 95% CI.

Finally, analysis using Crystal Miner software is user-friendly but is not available for diagnostic procedures, such as chimerism analysis. Interestingly, genotyping and quantification by GenDx reagents can be performed with this method without adaptation, even if a change in the ORG560 or RED610 fluorophore in the GenDx KMRtype kit would be desirable to optimize detection.

The Devyser for NGS method reduces technical stages, manages informative markers, reduces stockholding and reagent waste, and performs the screening and monitoring of chimerism in a single experiment. The number of quantification points depends on the flow cell type and minimum coverage needed, varying from 11 quantification samples for MiSeq v2 Micro (300 cycles) to 60 quantification samples for MiSeq v3 (150 cycles). In addition, evaluation of the

associated software brings new perspectives regarding the flexibility of the choice of informative markers and longitudinal patient follow-up, particularly according to cell population. Furthermore, this technique can be automated and easily integrated into the laboratory using NGS Illumina technology.

Finally, the correlation and concordance between the results obtained by the 2 methods were very high, as revealed by regression and Bland-Altman analysis, indicating that these 2 chimerism methods can be readily implemented in routine clinical diagnostics. Thus, whereas NGS is designed for large series, cdPCR, given its flexibility (eg, single consumable ready to use, easy storage, results in <4 hours), can be used in control or emergency circumstances. In addition, chimerism analysis for both methods was performed with low amounts of DNA, a

common condition early after transplantation (usually until day +30), when HSCT recipients have a low white blood cell count.

A limitation of this study is the low number of HSCT recipients. Further validation studies are needed to integrate cdPCR or NGS in clinical routines for chimerism monitoring.

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