



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

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**Evaluation of next-generation sequencing and Crystal Digital PCR™ for chimerism monitoring of post-allogeneic haematopoietic stem cell transplantation.**

Pascal Pedini<sup>1,2</sup>, Nicem Cherouat<sup>1</sup>, Agnes Basire<sup>1</sup>, Sophie Simon<sup>1</sup>, Laurène Budon<sup>1</sup>, Monique Pourtein<sup>3</sup>, Sandra Grondin<sup>4</sup>, Philippe Moskovtchenko<sup>4</sup>, Jacques Chiaroni<sup>2</sup>, Gérard Michel<sup>5</sup>, Coralie Frassati<sup>1,2</sup>, Christophe Picard<sup>1,2</sup>.

1. Immunogenetic Laboratory, EFS PACC, Marseille

2. Aix Marseille Univ, CNRS, EFS, ADES, Marseille

3. Immunogenetic Laboratory, CHU Nice

4. Immunogenetic Laboratory, EFS AURA, Lyon

5. Department of Pediatric Hematology Oncology, Marseille

**Corresponding author:**

Corresponding author: Dr. Pascal Pedini, PharmD, Immunogenetic Laboratory - Établissement Français du Sang PACA-Corse, 149 Bd Baille 13005 France. Tel: +33 (0) 4 91 18 95 96. Fax: +33 (0) 4 91 48 16 02. [Pascal.pedini@efs.sante.fr](mailto:Pascal.pedini@efs.sante.fr)

**Running Title:** Chimerism quantification by NGS and Crystal Digital PCR™

**Keywords:** chimerism, haematopoietic stem cell transplantation, Crystal Digital PCR™, NGS

**Abbreviations:**

HSCT: haematopoietic stem cell transplantation; STR: short tandem repeats; qPCR: quantitative PCR; dPCR: digital PCR; ddPCR: droplet digital PCR; cdPCR: Crystal Digital PCR™; NGS: next-generation sequencing; MRD: minimal residual disease; CE-IVD: European Conformity for *In Vitro* Diagnostics; EPT: external proficiency testing

**Conflicts of Interest:** The authors declare no conflicts of interest.

**Abstract:**

Haematopoietic stem cell transplantation (HSCT) is a curative treatment for most haematological diseases. To evaluate the level of donor engraftment, chimerism must be carefully monitored after HSCT. Short tandem repeats, quantitative PCR and, more recently, digital PCR are widely used to determine the proportions of donor/recipient cells after HSCT. Presently, the screening and quantification of chimerism have been evaluated by two new methods, a ready-to-use NGS-based method using the “Devyser®ChimerismNGS” kit and an original combination of the Stilla® Crystal Digital PCR™ platform (cdPCR) with 3-colour 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 testing showed the reliability of the results (0.1%–50%) for the two methods. They are also highly sensitive (0.1%) and accurate. The values for chimerism of these two methods are correlated and concordant with those of the reference methods. Additionally, the software Advyser (Devyser®) is user friendly and remarkably adapted to chimerism monitoring. In conclusion, these two new and innovative methods are easy and user friendly in all molecular, haematology and immunogenetic laboratories and allow the genotyping and monitoring of chimerism with high performance and sensitivity.

## **Introduction:**

Haematopoietic stem cell transplantation (HSCT) is a curative treatment for most haematological 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 but also post-HSCT to detect the risk of disease relapse in malignant disease (1,2). Currently, two historical methods, short tandem repeat (STR) PCR and qPCR (quantitative PCR) methods, are routinely performed in laboratories. However, their ranges of analysis—i.e., 5%–80% (STR) and 0.1%–30% (qPCR)—and the need for two technical steps, genotyping of genetic markers of the donor/recipient pair and then quantification of chimerism using informative recipient markers, are limitations of their applications. More recently, we and others have shown that droplet digital PCR Bio-Rad® (ddPCR) using chimerism quantification kits, specific or not for this ddPCR method, marked or not CE-IVD, is an effective alternative. The quantification ranges of this method were at least between 0.1% and 90% (3–5). Among the advantages of ddPCR compared with 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 end point of amplification, resulting in a lower sensitivity of PCR inhibitors. However, the genotyping step is difficult to be integrated and, therefore, must be performed using a qPCR platform. Stilla Technologies® has developed the first digital PCR system with 3-colour multiplexing capacity, named the Naica System. It performs digital PCR using a hybrid approach, named Crystal Digital PCR™ (cdPCR), combining a digital PCR chamber, which relies on 2D arrays of microchambers to partition the sample, and the use of partitions as implemented in ddPCR (6). This multiplexing Crystal Digital PCR can decrease the number of wells used to identify informative markers for HSCT pairs.

NGS technologies have revolutionised the field of genomics, and their applications have been extended to different fields, such as clinical diagnostics and forensic science (7,8). In this context, few custom NGS chimerism panels using single-nucleotide polymorphism (SNP) markers have been applied to chimerism and have shown a close concordance with historical results from STR analysis. Their sensitivity has reached 0.5%–1%, which is higher than 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 many 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 lasts between 36 hours and 48 hours. Recently, a few commercial kits have offered an NGS method for chimerism as a complete workflow solution for laboratories, combining a reliable testing process with a designed for-purpose analytical software.

Thus, this study aimed to evaluate two 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). First, we evaluated the capacity of these two methods to identify informative markers for HSCT pairs to be used in chimerism monitoring. Second, we evaluated whether these two methods are accurate and sensitive to monitor chimerism by quantitative determination of mixed chimerism using an artificial DNA mixture and DNA from HSCT patients.

## **Materials and methods:**

**Biological samples:**

Artificial chimeric samples were prepared from the donor samples of two healthy subjects (1 man and 1 woman). 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%.

In total, 43 samples from 13 patients with haematologic malignant diseases treated with allo-HSCT were included in this study (table 1). The underlying diseases were immunodeficiency (ID; n = 3), thalassemia (Th; n = 1); acute myeloid leukaemia (AML; n = 3), acute lymphoblastic leukaemia (ALL, n = 5) and myelofibrosis (MF, n = 1). Two samples were collected at the time of acute leukaemia relapse. The chimerism of every sample was previously quantified by STR analysis using the AmpFLSTR™ Identifiler™ PCR Amplification Kit (Applied Biosystems, Warrington, UK) and/or Q-PCR using the Jeta Molecular kit (JETA Molecular BV, Utrecht, The Netherlands).

For comparison between different techniques, 17 samples (15 whole blood, 1 CD3 isolation and 1 bone marrow) were from 6 paediatric patients and 3 samples (0.1%, 2%, 10% chimerism expected) were from an external quality control “SFHI EPT” (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 Laboratories®, Hercules, CA, USA) using a specific probe for the Y chromosome (KMR54 (GenDX®)) and a non-polymorphic endogenous gene (RPP30) (5).

For the other samples, 26 samples (20 whole blood, 3 bone marrow, 2 CD3 and 1 CD33 isolation) were from 7 patients (4 adult and 3 paediatric patients). Sixteen chimerism analyses from 11 STR and 5 Q-PCR samples were performed by NGS, and 10 chimerism analyses from 2 STR and 8 Q-PCR 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 records.

#### 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 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, USA). All the DNA used in the study had absorbance ratios of  $A_{260}/A_{280} > 1.8$  and  $A_{260}/A_{230} > 1.8$ . The DNA samples were stored at  $-40^{\circ}\text{C}$  for less than 48 months before performing experiments.

#### Chimerism analysis by NGS:

The principle and technical characteristics of NGS are described in table 2.

The chimerism level was quantified using the Devyser® Chimerism kit 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 the chimerism status followed by specific Illumina NGS library preparation and sequencing. The resulting sequences are analysed using the "ADVYSER for Chimerism" software 1.3 version. This software performs the following: 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 %VAF (variant allele frequency) 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:  $\text{Ref reads} / (\text{Ref reads} + \text{Alt reads})$ . Pre-transplant 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 (+/+ vs -/- or -/- vs +/+ and +/- vs +/+ or -/-, respectively). All the informative markers are automatically selected, 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 (i.e., a post-transplant sample is imported instead of a pre-transplant sample), with sample impurity (i.e., 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 non-optimal run, index-hopping or carry-over events). For monitoring, there are two quality criteria (coverage and noise); for each, there are two levels of warning (“yellow” and “red”). The optimal coverage depends on the % chimerism. A coverage > 10,000 reads is required to call chimerism at 0.1% with high precision and sensitivity. If the detected % chimerism is higher (>1%), 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 digital PCR (Crystal Digital PCR™) platform Naica™ System (Stilla Technologies®) with a 3-colour target multiplexing capability (detection



wavelengths: 495–520 nm, 560–610 nm, 655–720 nm). Pre-transplantation genotyping and chimerism quantification were performed using GenDX® KMRtype and KMRtrack (KMR54: Y-linked probe) CE-IVD qPCR markers, respectively. For one sample, we used 10 µL of Perfecta 2X UNG, 2.50 µL of fluorescein (1 µM), 6.75 µL of 5X GenDX® KMR54 FAM, 1.35 µL of 20X RPP30 gene control HEX and 6 µL of 25 ng/µL of 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°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds and 60°C for 15 seconds. Image acquisition was performed using the Naica Prism3 reader and 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 analysed 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 the 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 software (2.1.6 version) automatically calculates and sets an optimal threshold to discriminate between positive and negative drops. This threshold can be manually modified. Based on the chosen fluorescence threshold, the software automatically calculates the concentration of target nucleic acid for each fluorescence, as well as 95% confidence intervals. The results are displayed in a table or graphs. The GenDX® KMRtype kit comprises 39 individual markers with 13 multiplexing PCR mixes, using 3 probes by mix carrying FAM, ORG560 and RED610 as dyes. The probes carrying the FAM fluorophore were detected in the 495- to 520-nm wavelength window, ORG560 in the 560- to 610-nm wavelength window and RED610 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 more than 90% of informative probes usually used in our laboratory.

For quantification, the informative “recipient” or “donor” marker has a FAM fluorescence and the non-polymorphic gene (RPP30) has a HEX fluorescence. Quantification of donor or recipient chimerism is obtained by the ratio of the number of droplets: informative target/non-polymorphic target (X2). The limit of blank (LOB) and limit of detection (LOD) were assessed using three 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’s 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 both 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 is FAM (Table 3).

#### 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 for the whole range was  $R^2=0.999$  ( $p<0.001$ ) (fig. 1a) and  $R^2= 0.995$  ( $p<0.001$ ) for low values (from 0.1% to 1%) (fig. 1b). The CVs of reproducibility were  $< 20\%$  for all recipient chimerism percentages (fig. 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 patients. Three to 11 informative polymorphisms were automatically identified for each donor/recipient pair, producing the expected percentage of chimerism. If the laboratory selection rules are applied—i.e., the SNP was present exclusively in the donor or recipient in the homozygous or heterozygous state (+/+ vs -/- or +/- vs -/-), similar results to automatic rules were obtained (Table 4). Rare cases were identified with the “Background noise” warning, and only one case had the “Unexpected VAF” warning (EPT sample) with no impact on the result of chimerism quantification.

#### Analytical Performance of the 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 for the whole range was  $R^2=0.999$  ( $p<0.001$ ) (fig. 1a) and  $R^2= 0.974$  ( $p<0.001$ ) for low values (from 0.1% to 1%) (fig. 1b). The CVs of reproducibility were  $< 20\%$  for all chimerism percentages (fig. 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 non-conforming quality criteria were observed.

#### Comparison of chimerism quantification

Chimerism quantification by cdPCR and NGS methods were correlated more strongly with the ddPCR technique (fig. 3a). The correlation coefficient for the cdPCR method was  $R^2=0.995$  ( $p<0.001$ ) and that for the NGS method was  $R^2=0.997$  ( $p<0.001$ ). The Bland–Altman plot confirmed a concordance of the results for % chimerism in cdPCR and NGS with ddPCR (fig. 4). Chimerism quantification by the NGS (fig. 3b) and cdPCR (fig. 3c) methods are correlated more strongly with the qPCR and STR techniques.

#### **Discussion:**

This study evaluated two new methods, an original combination of Crystal Digital PCR™ 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, Crystal Digital PCR™ is a system with 3-colour 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 three dyes of this kit are not completely adapted to their use by Crystal Digital PCR™ because the ORG560 and RED610 fluorophores have wavelengths that are located at the end of the 2nd signal of the Naica platform. Manual compensation was performed to obtain a more specific signal for these two fluorophores. Thus, no assignment error, negative or positive, was found for these two fluorophores in the other cases. The only two polymorphism identification errors involved inexplicably two different probes associated with the FAM fluorophore. These probes had

been at least once positive and negative without error of identification for the other genotyping. The design of these probes may 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 Advyser software. Each pair had at least three donor or recipient informative markers identified. Manual changes, considering homozygous donor or recipient polymorphisms only as informative, did not alter the quantification results of chimerism, highlighting the right choice of automatic selection of informative polymorphisms. Interestingly, the Devyser® panel comprises markers with a low bias from ethnic parameters, allowing robust discrimination when analysing HLA identical siblings.

Our study showed that these two methods had very similar analytical capacities for chimerism quantification. Both technical approaches showed a similar sensitivity (i.e., 0.1%), a range of analysis from 50% to 0.1% and a reproducibility with a CV < 20%. Additionally, they allowed the accurate identification of quantitative differences in the presence of low levels of chimerism. The quantitative results showed close concordance with the results from droplet digital PCR (Bio-Rad®), providing validation of these two methods in chimerism monitoring.

Thus, quantification of chimerism by cdPCR is a sensitive and accurate method (i.e., no requirement for standard curves, efficiency controls and multiple replicates as qPCR) to monitor HSCT patients. By performing all stages of digital PCR in a single consumable ready to use, the Naica system offers a quick and easy solution. Moreover, this method is adapted to clinical emergencies (results in < 4 h). Interestingly, the droplets in the chip can be stored for up to 8 days at room temperature before reading. This advantage has made 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.

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). Additionally, evaluation of the associated software brings new perspectives regarding the flexibility of the choice of informative markers and longitudinal follow-up of the patient, 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 two methods were very high, as revealed by regression and Bland–Altman analysis, indicating that these two chimerism methods can be readily implemented in routine clinical diagnostics. Thus, while NGS is designed for large series, cdPCR, by its flexibility of use (e.g., single consumable ready to use, easy storage and results in <4 h), can be used in controls or emergency. Additionally, chimerism analysis for both methods was performed with low amounts of DNA, a common condition early after transplantation (usually until day +30) when HSCT patients have a low white blood cell count.

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

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313 **Conflicts of Interest:** The authors declare no conflicts of interest.

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## Figure Legends:

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. (1a: whole range; 1b: low chimerism < 1%)

Figure 2: Reproducibility of the cdPCR and NGS methods. Each % chimerism was tested on 3 different experiments to calculate the CV of each method.

Figure 3: Correlation of the % chimerism results between cdPCR and ddPCR and between NGS and ddPCR using regression curve analysis (3a). Correlation of the % chimerism results between NGS and qPCR/STR using regression curve analysis (3b). Correlation of the % chimerism results between cdPCR and qPCR/STR using regression curve analysis (3c).

Figure 4: Concordance of the % chimerism results between cdPCR and ddPCR (4a) and between NGS and ddPCR (4b) 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 revealed that all data points were within the 95% CI.

369 **Tables:**

370 Table 1: Information of the allo-SCT patients included in the study and their clinical and chimerism  
 371 (% recipient) follow-up

Patient ID	Sample	Indication	Time after transplantation (Days)	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%	

372

373 WB: whole blood/BM: bone marrow/AML: acute myeloid leukaemia/ALL: acute lymphoblastic leukaemia.

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		cdPCR	NGS
Principle		<ul style="list-style-type: none"> <li>- Selection of donor/recipient informational markers</li> <li>- DNA and marker compartmentalisation in thousands of microreactors</li> <li>- Amplification of microreactors</li> <li>- Absolute quantification by digital reading (0 or 1)</li> <li>- Chimerism calculation by the informative marker/reference ratio</li> </ul>	<ul style="list-style-type: none"> <li>- Multiplex PCR amplification of 24 informative markers (ins/del -75 bp), distributed on 17 autosomal chromosomes &gt; one tube per sample</li> <li>- Preparation of an Illumina-compatible library: amplicon type &gt; one tube per sample</li> <li>- Paired-end sequencing 2x75 bp</li> <li>- Automatic data analysis (genotyping and monitoring) and longitudinal follow-up of patient records</li> </ul>
Genotyping		Independent experience in addition to quantification	Same protocol as quantification
	Duration	Cf Monitoring	Cf Monitoring
	Number of markers	21 (in triplex)	24
	DNA Quantity	10 ng per well	60 ng
	Number of points	A 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) – 1:00 (Library prep) Instrument times: 1:45 (Amplification) - 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	+	-

Table 2: Principle and technical characteristics of cdPCR and NGS

379 Table 3: Comparison of HSCT genotyping by cdPCR and by qPCR using the GenDX® KMRtype kit

380

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.

381 **Table 4:** Comparison of the % chimerism results from NGS according to the automatic and manual  
382 selection criteria of the informative markers

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

383

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. (1a: whole range; 1b: low chimerism < 1%)

Figure 1a : whole range

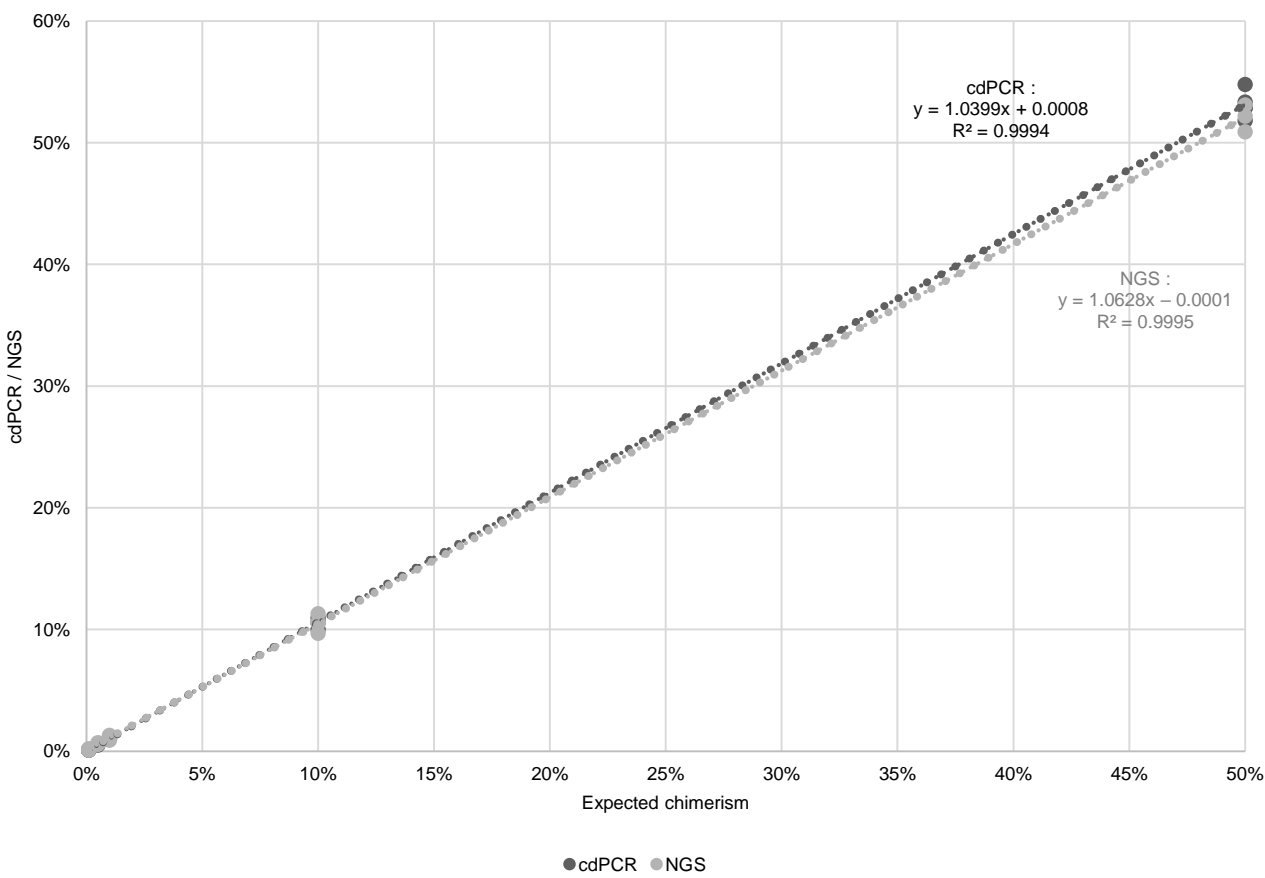


Figure 1b : low chimerism < 1%

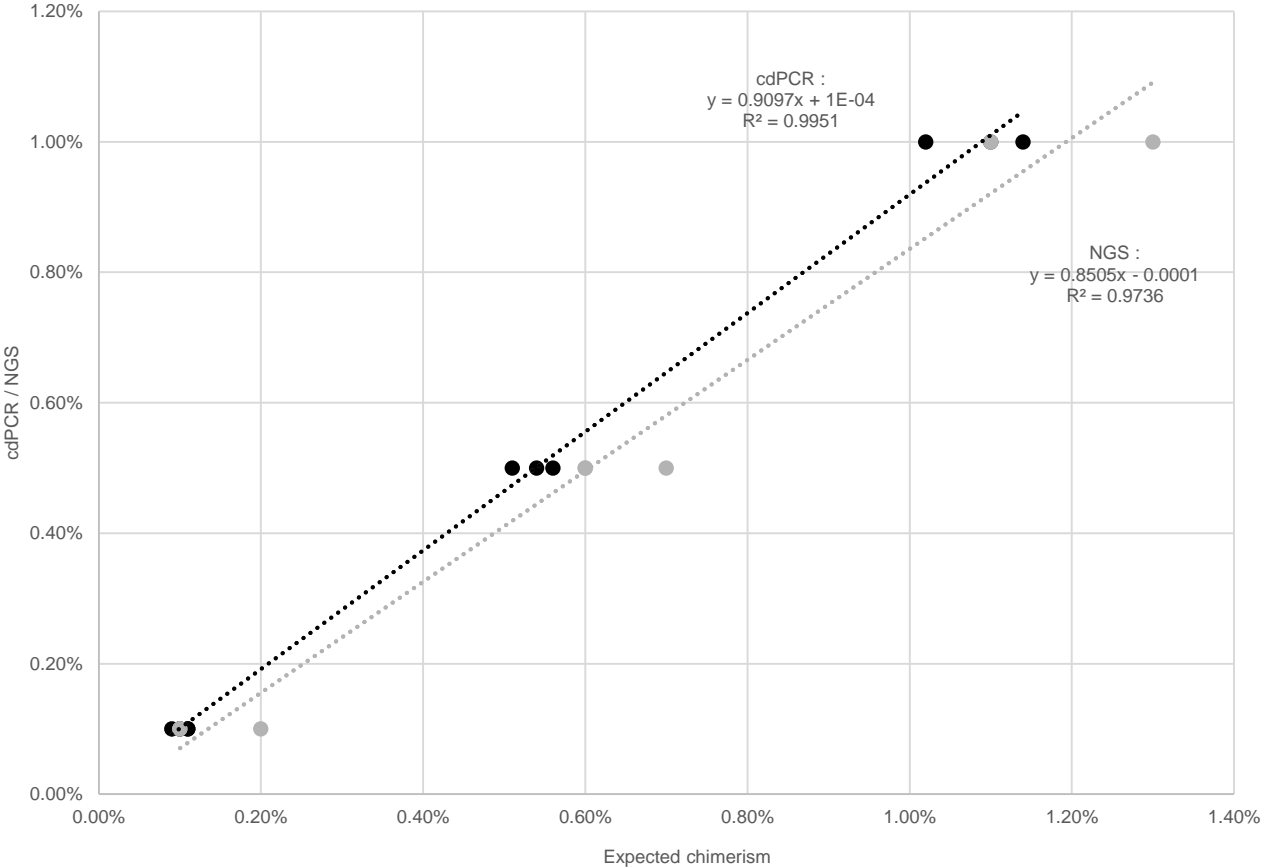




Figure 2: Reproducibility of cdPCR and NGS methods. Each % chimerism was tested on 3 different experiments to calculate the CV of each method.

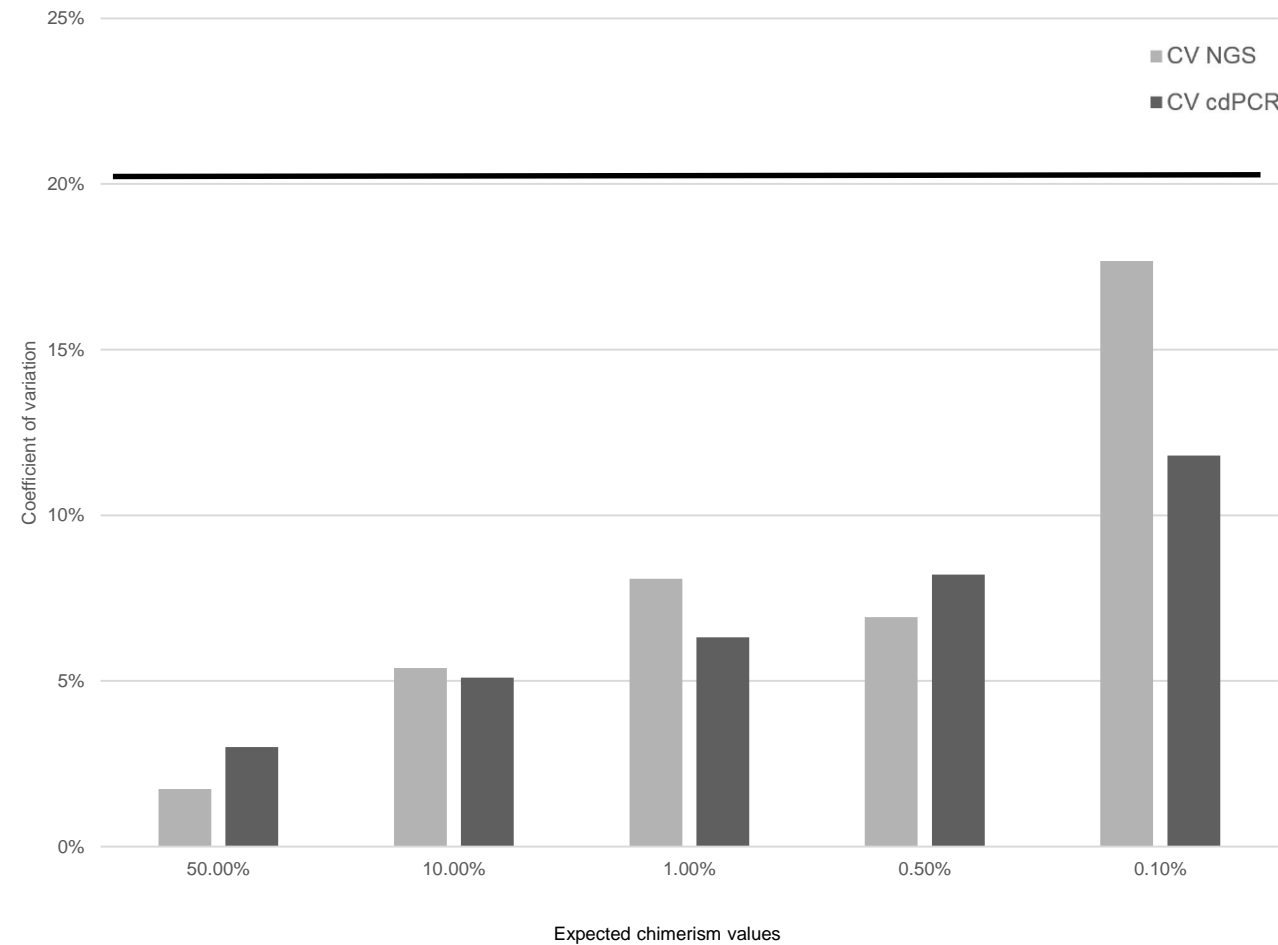


Figure 3a: Correlation of % chimerism results between cdPCR and ddPCR and between NGS and ddPCR, using regression curve.

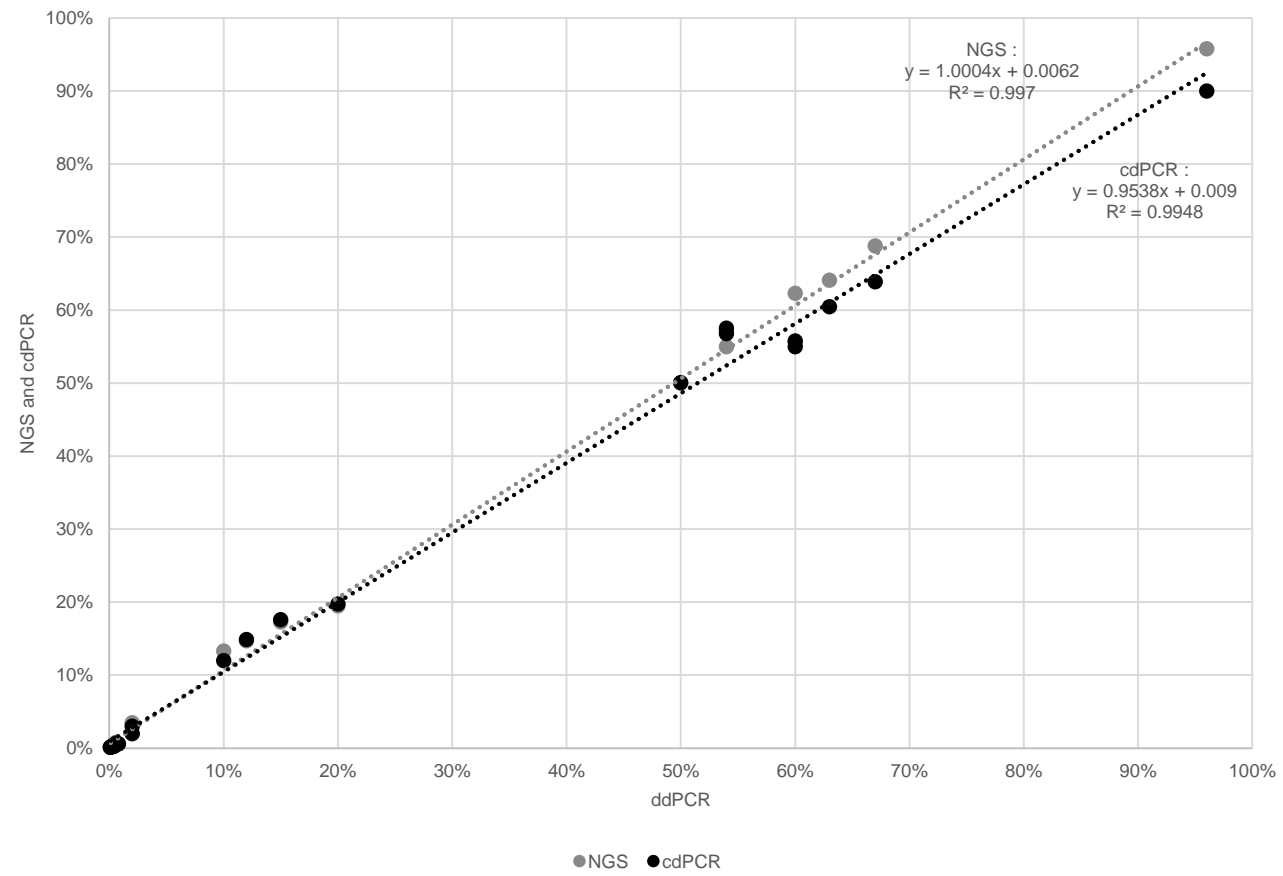


Figure 3b: Correlation of % chimerism results between NGS and qPCR / STR, using regression curve.

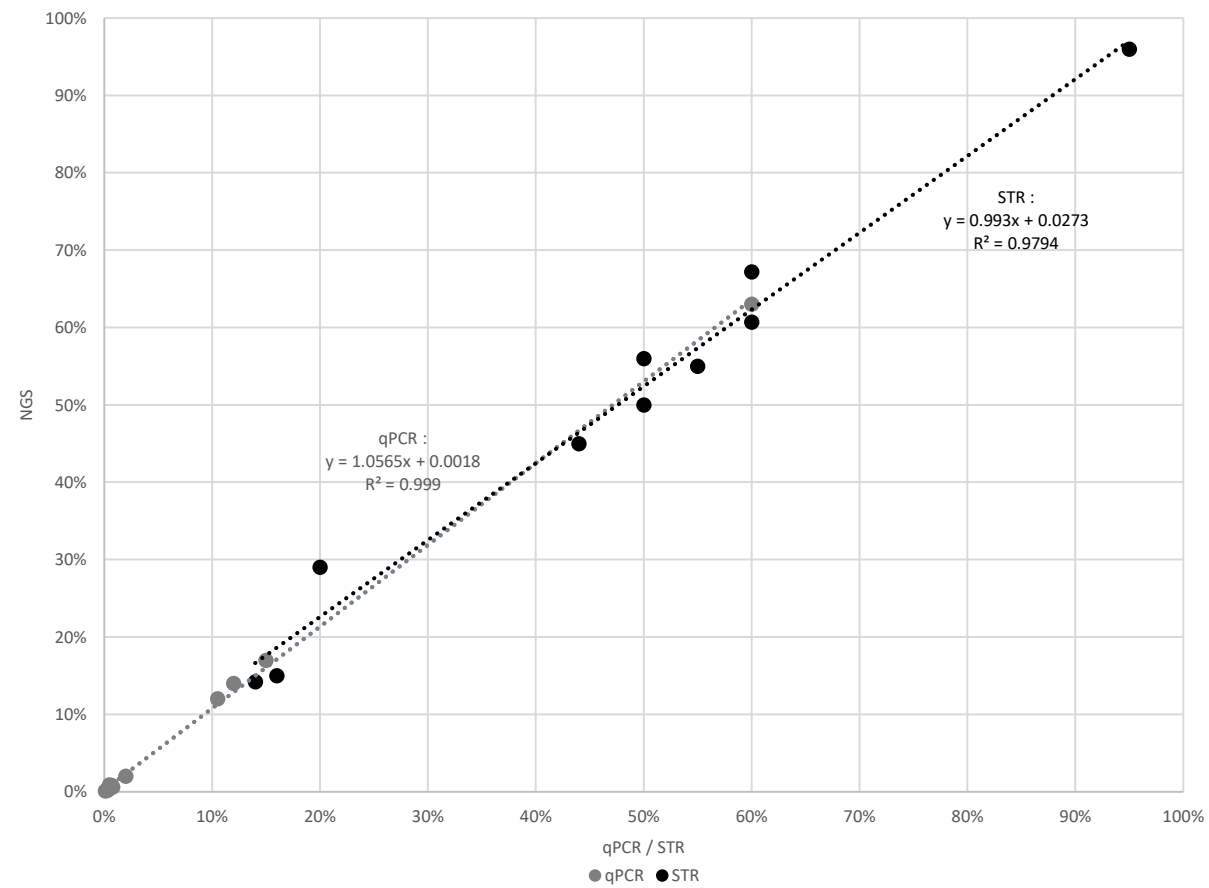


Figure 3c: Correlation of % chimerism results between cdPCR and qPCR / STR, using regression curve.

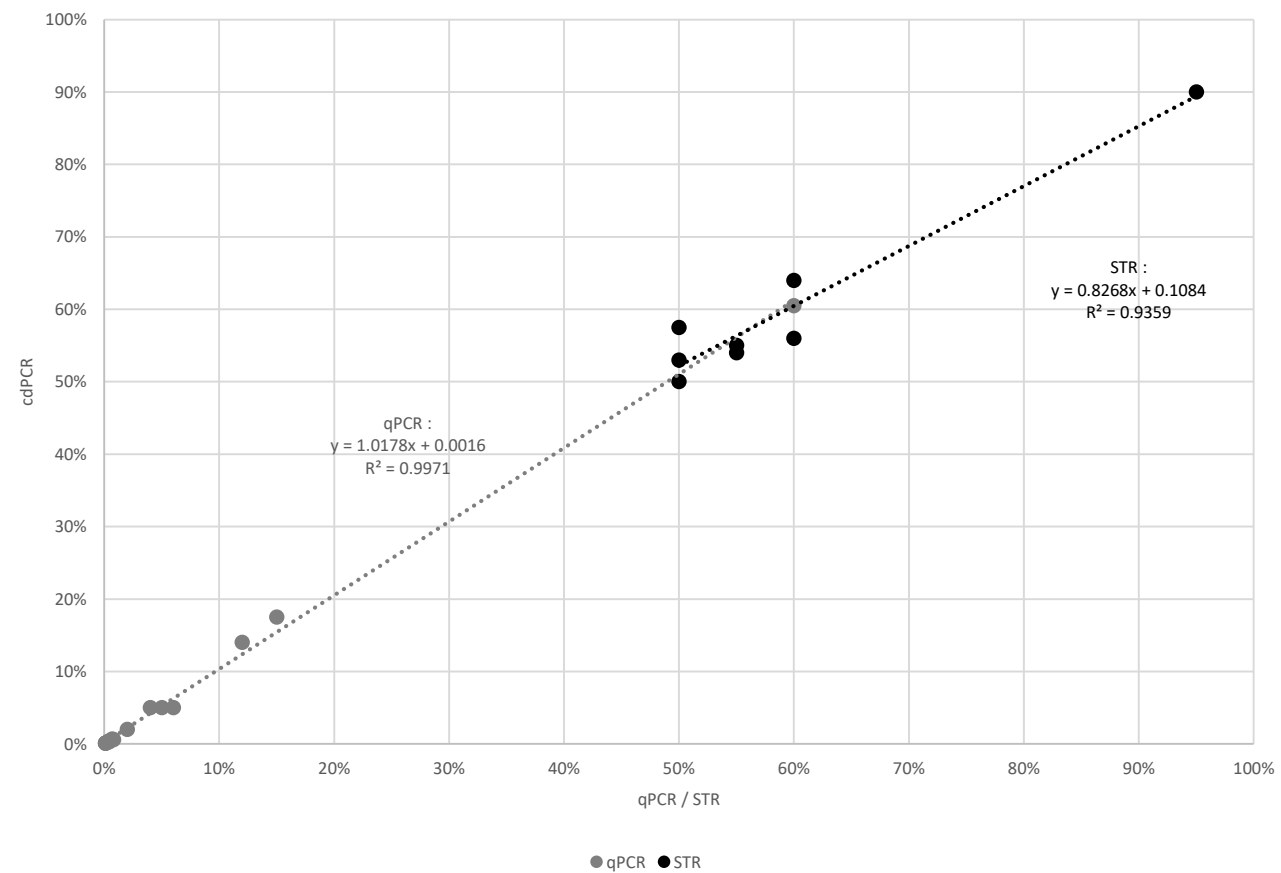


Figure 4: Concordance of % chimerism results between cdPCR and ddPCR (4a) using Bland-Altman diagram

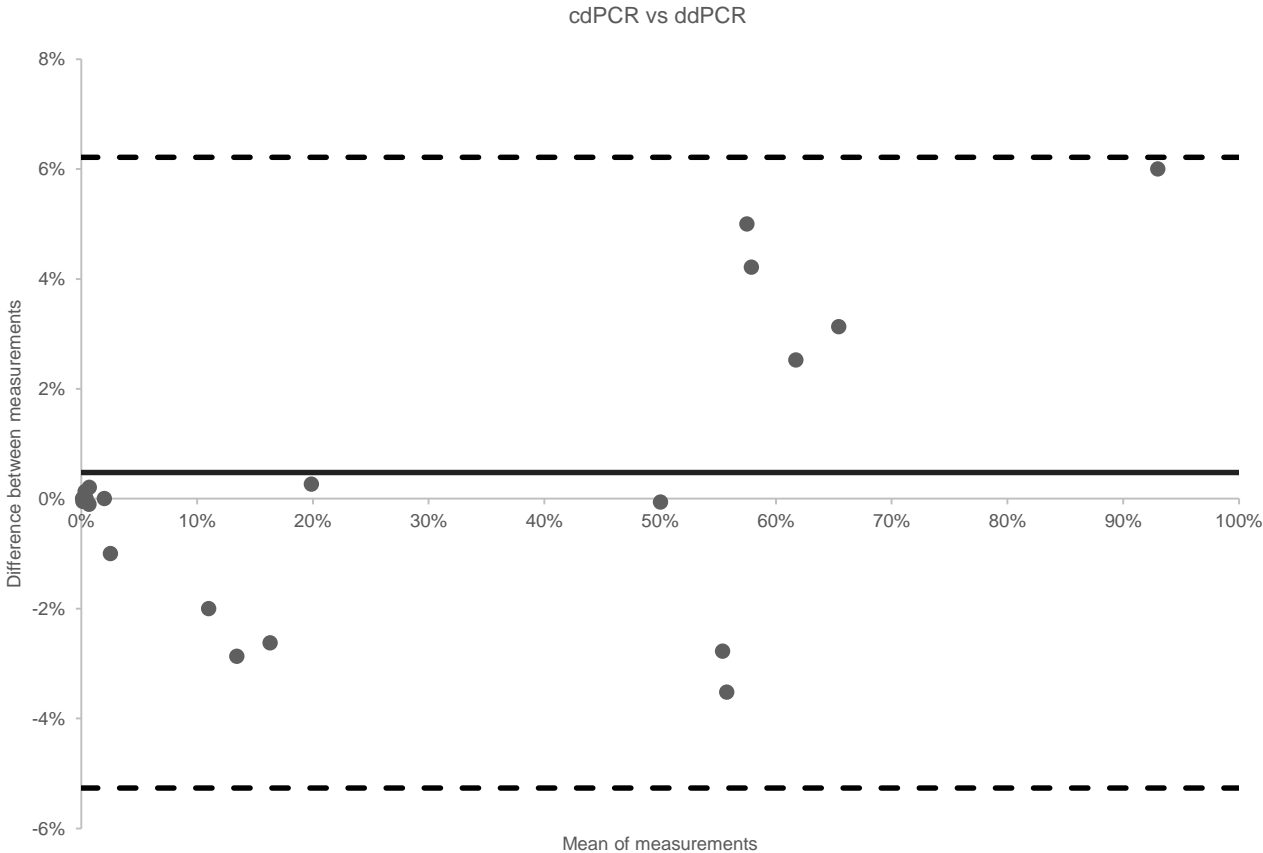


Figure 4: Concordance of % chimerism results between NGS and ddPCR (4b), using Bland-Altman diagram

