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

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

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

18 **Abbreviations:**

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

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

24 **Abstract:**

25 Haematopoietic stem cell transplantation (HSCT) is a curative treatment for most
26 haematological diseases. To evaluate the level of donor engraftment, chimerism must be
27 carefully monitored after HSCT. Short tandem repeats, quantitative PCR and, more recently,
28 digital PCR are widely used to determine the proportions of donor/recipient cells after HSCT.
29 Presently, the screening and quantification of chimerism have been evaluated by two new
30 methods, a ready-to-use NGS-based method using the “Devyser®ChimerismNGS” kit and an
31 original combination of the Stilla® Crystal Digital PCR™ platform (cdPCR) with 3-colour
32 multiplexing capacity using GenDX®KMRtrack reagents. The genotyping of 4 HSCT pairs
33 by cdPCR using 11 triplex mixes of the GenDX®KMRtype kit was consistent at 98.8% with
34 qPCR. Informative samples (n=20) from 6 donor/recipient pairs and 1 external proficiency
35 testing showed the reliability of the results (0.1%–50%) for the two methods. They are also
36 highly sensitive (0.1%) and accurate. The values for chimerism of these two methods are
37 correlated and concordant with those of the reference methods. Additionally, the software
38 Advyser (Devyser®) is user friendly and remarkably adapted to chimerism monitoring. In
39 conclusion, these two new and innovative methods are easy and user friendly in all molecular,
40 haematology and immunogenetic laboratories and allow the genotyping and monitoring of
41 chimerism with high performance and sensitivity.

42 **Introduction:**

43 Haematopoietic stem cell transplantation (HSCT) is a curative treatment for most
44 haematological diseases. To evaluate the level of donor engraftment, chimerism must be
45 carefully monitored after HSCT. Chimerism monitoring is very important in the engraftment
46 phase to detect graft failure but also post-HSCT to detect the risk of disease relapse in
47 malignant disease (1,2). Currently, two historical methods, short tandem repeat (STR) PCR
48 and qPCR (quantitative PCR) methods, are routinely performed in laboratories. However,
49 their ranges of analysis—i.e., 5%–80% (STR) and 0.1%–30% (qPCR)—and the need for two
50 technical steps, genotyping of genetic markers of the donor/recipient pair and then
51 quantification of chimerism using informative recipient markers, are limitations of their
52 applications. More recently, we and others have shown that droplet digital PCR Bio-Rad®
53 (ddPCR) using chimerism quantification kits, specific or not for this ddPCR method, marked
54 or not CE-IVD, is an effective alternative. The quantification ranges of this method were at
55 least between 0.1% and 90% (3–5). Among the advantages of ddPCR compared with qPCR
56 are that ddPCR enables the multiplexed absolute quantification of the target and endogenous
57 genes in the same assay and does not require a standard curve. Furthermore, ddPCR detects
58 the target at the end point of amplification, resulting in a lower sensitivity of PCR inhibitors.
59 However, the genotyping step is difficult to be integrated and, therefore, must be performed
60 using a qPCR platform. Stilla Technologies® has developed the first digital PCR system with
61 3-colour multiplexing capacity, named the Naica System. It performs digital PCR using a
62 hybrid approach, named Crystal Digital PCR™ (cdPCR), combining a digital PCR chamber,
63 which relies on 2D arrays of microchambers to partition the sample, and the use of partitions
64 as implemented in ddPCR (6). This multiplexing Crystal Digital PCR can decrease the
65 number of wells used to identify informative markers for HSCT pairs.

66 NGS technologies have revolutionised the field of genomics, and their applications have been
67 extended to different fields, such as clinical diagnostics and forensic science (7,8). In this
68 context, few custom NGS chimerism panels using single-nucleotide polymorphism (SNP)
69 markers have been applied to chimerism and have shown a close concordance with historical
70 results from STR analysis. Their sensitivity has reached 0.5%–1%, which is higher than that
71 of STR analysis (9,10). These panels are characterized by many selected SNPs, which have a
72 heterozygosity frequency of approximately 0.5 for the European population to obtain many
73 informative markers for each HSCT pair and are located in different regions of each
74 chromosome, thus avoiding false-negative results caused by chromosomal deletions during
75 relapses of a few malignancies (11). Moreover, the genotyping of genetic markers of
76 donor/recipient pairs and quantification of chimerism are performed in the same protocol,
77 which lasts between 36 hours and 48 hours. Recently, a few commercial kits have offered an
78 NGS method for chimerism as a complete workflow solution for laboratories, combining a
79 reliable testing process with a designed for-purpose analytical software.

80 Thus, this study aimed to evaluate two new chimerism quantification methods, Crystal Digital
81 PCR™ (Stilla Technologies®, Villejuif, France) using KMR kits (GenDX®, Utrecht, the
82 Netherlands) and NGS using the Devyser Chimerism kit (Devyser AB®, Hägersten, Sweden).
83 First, we evaluated the capacity of these two methods to identify informative markers for
84 HSCT pairs to be used in chimerism monitoring. Second, we evaluated whether these two
85 methods are accurate and sensitive to monitor chimerism by quantitative determination of
86 mixed chimerism using an artificial DNA mixture and DNA from HSCT patients.

87

88 **Materials and methods:**

89 Biological samples:

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

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

101

102 For comparison between different techniques, 17 samples (15 whole blood, 1 CD3 isolation
103 and 1 bone marrow) were from 6 paediatric patients and 3 samples (0.1%, 2%, 10%
104 chimerism expected) were from an external quality control “SFHI EPT” (Société
105 Francophone Histocompatibilité et Immunogénétique, External Proficiency Testing). These
106 samples were selected for their informativeness concerning the Y chromosome. Chimerism
107 was previously evaluated by ddPCR (Bio-Rad Laboratories®, Hercules, CA, USA) using a
108 specific probe for the Y chromosome (KMR54 (GenDX®)) and a non-polymorphic
109 endogenous gene (RPP30) (5).

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

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

117

118 DNA isolation methods:

119 The genomic DNA of the donors (peripheral whole blood) and recipients (whole blood, CD3
120 and CD33 isolation and bone marrow) were isolated using the Quickgene-610 L system
121 (Kurabo Industries®, Osaka, Japan) with a DNA Blood kit. The DNA concentration and
122 purity were checked by absorbance measurement using an ND-One spectrophotometer
123 (Nanodrop Technologies®, Wilmington, DE, USA). All the DNA used in the study had
124 absorbance ratios of $A_{260}/A_{280} > 1.8$ and $A_{260}/A_{230} > 1.8$. The DNA samples were stored
125 at -40°C for less than 48 months before performing experiments.

126

127 Chimerism analysis by NGS:

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

129 The chimerism level was quantified using the Devyser® Chimerism kit following the
130 manufacturer's instructions. This kit is based on multiplex PCR using a range of 24 highly
131 informative markers distributed over 17 chromosomes suitable for the screening of a
132 recipient/donor pair and monitoring of the chimerism status followed by specific Illumina
133 NGS library preparation and sequencing. The resulting sequences are analysed using the
134 "ADVYSER for Chimerism" software 1.3 version. This software performs the following:
135 identification of donor- and recipient-specific markers, calculation of the chimerism
136 percentage, automatic generation of trending charts to assess monitoring results, and parallel
137 trending of various cell populations.

138 Genotyping is determined by the %VAF (variant allele frequency) calculation for each marker
139 using the number of reads classified as “reference” (Ref) and “alternative” (Alt) of the
140 reference sequence hg19 (human genome 19; Genome Reference Consortium). The %VAF is
141 calculated as follows: $\text{Ref reads}/(\text{Ref reads} + \text{Alt reads})$. Pre-transplant samples are expected
142 to have a VAF close to 0% (-/-), 50% (+/-) or 100% (+/+). A marker is informative if the
143 recipient and donor are homozygous for opposite genotypes or if the recipient is heterozygous
144 and the donor is homozygous (+/+ vs -/- or -/- vs +/+ and +/- vs +/+ or -/-, respectively). All the
145 informative markers are automatically selected, but the user can deselect them manually.
146 Three warnings should be considered for the selection of the markers: “low coverage”, when
147 the minimum recommended coverage of 100 reads/marker is not met; “unexpected VAF”,
148 when the %VAF is between 1% and 40% or between 60% and 99% (due to import mix-up
149 (i.e., a post-transplant sample is imported instead of a pre-transplant sample), with sample
150 impurity (i.e., as detected in saliva swabs) or if the patient has had previous transplantations);
151 and “background noise”, when the VAF is between 0.1% and 1% or between 99% and 99.9%
152 (due to non-optimal run, index-hopping or carry-over events). For monitoring, there are two
153 quality criteria (coverage and noise); for each, there are two levels of warning (“yellow” and
154 “red”). The optimal coverage depends on the % chimerism. A coverage > 10,000 reads is
155 required to call chimerism at 0.1% with high precision and sensitivity. If the detected %
156 chimerism is higher (>1%), a coverage of > 1,000 reads/marker is sufficient to determine the
157 % chimerism with high precision and sensitivity.

158

159 Chimerism analysis by cdPCR:

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

161 Chimerism was quantified using the digital PCR (Crystal Digital PCR™) platform Naica™

162 System (Stilla Technologies®) with a 3-colour target multiplexing capability (detection

163 wavelengths: 495–520 nm, 560–610 nm, 655–720 nm). Pre-transplantation genotyping and
164 chimerism quantification were performed using GenDX® KMRtype and KMRtrack (KMR54:
165 Y-linked probe) CE-IVD qPCR markers, respectively. For one sample, we used 10 μ L of
166 Perfecta 2X UNG, 2.50 μ L of fluorescein (1 μ M), 6.75 μ L of 5X GenDX® KMR54 FAM,
167 1.35 μ L of 20X RPP30 gene control HEX and 6 μ L of 25 ng/ μ L of DNA (150 ng). The PCR
168 mix was pipetted into each of 4 inlet ports of Sapphire chips. Three Sapphire chips were
169 positioned into the Naica Geode, which was programmed to perform the sample partitioning
170 step, followed by the PCR thermal cycling program: 95°C for 10 minutes, followed by 45
171 cycles of 95°C for 10 seconds and 60°C for 15 seconds. Image acquisition was performed
172 using the Naica Prism3 reader and the following exposure times: blue signal: 100 ms; green
173 signal: 50 ms; red signal: 50 ms. cdPCR analysis was carried out in 2 h and 30 minutes. The
174 data were analysed using Crystal Miner software, which allows visual and intuitive quality
175 control of the results by jointly displaying images of the drop crystals and corresponding point
176 plots. These quality controls will enable the checking of the uniform distribution of positive
177 partitions across the drop crystals and distinguishing true positives from false positives caused
178 by dust particles in the droplets. The droplet number must be > 18,000. Crystal Miner
179 software (2.1.6 version) automatically calculates and sets an optimal threshold to discriminate
180 between positive and negative drops. This threshold can be manually modified. Based on the
181 chosen fluorescence threshold, the software automatically calculates the concentration of
182 target nucleic acid for each fluorescence, as well as 95% confidence intervals. The results are
183 displayed in a table or graphs. The GenDX® KMRtype kit comprises 39 individual markers
184 with 13 multiplexing PCR mixes, using 3 probes by mix carrying FAM, ORG560 and
185 RED610 as dyes. The probes carrying the FAM fluorophore were detected in the 495- to 520-
186 nm wavelength window, ORG560 in the 560- to 610-nm wavelength window and RED610 in
187 the 655- to 720-nm wavelength window. In this study, we evaluated 21 markers in 11 triplex

188 mixes (mix 1 and mixes 4 to 13), representing more than 90% of informative probes usually
189 used in our laboratory.

190 For quantification, the informative “recipient” or “donor” marker has a FAM fluorescence and
191 the non-polymorphic gene (RPP30) has a HEX fluorescence. Quantification of donor or
192 recipient chimerism is obtained by the ratio of the number of droplets: informative target/non-
193 polymorphic target (X2). The limit of blank (LOB) and limit of detection (LOD) were
194 assessed using three series, including 30 female samples and recording the number of false-
195 positive events for the Y-probe (KMR 54).

196

197 Statistical Analysis:

198 The square of the Pearson’s correlation coefficient (R^2 , p-value) was computed to determine
199 the replicability of the model (cdPCR, NGS and ddPCR). A Bland–Altman plot was used to
200 visualize the grade of concordance between the results of chimerism obtained by both cdPCR
201 and NGS methods.

202

203

204 **Results:**

205 Pretransplantation genotyping using cdPCR:

206 Of 168 markers tested (4 donor/recipient pairs) from 21 markers of 11 triplex mixes, 166
207 (98.8%) were consistent with those previously performed with qPCR. Only 2 markers in
208 different samples were negative, whereas the expected results were positive: the KMR33 and
209 KMR44 for both dyes is FAM (Table 3).

210

211 Analytical performance of cdPCR chimerism:

212 The limit of detection was achieved by testing the same female DNA 30 times with the
213 KMR54. The value of the detection limit was 0.663 copies/ μ L for 150 ng (12). Quantification
214 of chimerism by cdPCR was reliable over a wide range of measurements (from 0.1% to 50%,
215 3 points/%). The correlation coefficient for the whole range was $R^2=0.999$ ($p<0.001$) (fig. 1a)
216 and $R^2= 0.995$ ($p<0.001$) for low values (from 0.1% to 1%) (fig. 1b). The CVs of
217 reproducibility were $< 20\%$ for all recipient chimerism percentages (fig. 2). The sensitivity of
218 this method reached 0.1%.

219

220 Pretransplantation genotyping using NGS:

221 The screening of recipient and donor samples showed informative markers for all 6 HSCT
222 patients. Three to 11 informative polymorphisms were automatically identified for each
223 donor/recipient pair, producing the expected percentage of chimerism. If the laboratory
224 selection rules are applied—i.e., the SNP was present exclusively in the donor or recipient in
225 the homozygous or heterozygous state (+/+ vs -/- or +/- vs -/-), similar results to automatic
226 rules were obtained (Table 4). Rare cases were identified with the “Background noise”
227 warning, and only one case had the “Unexpected VAF” warning (EPT sample) with no impact
228 on the result of chimerism quantification.

229

230 Analytical Performance of the NGS Chimerism:

231 The quantification of chimerism by NGS was reliable over a wide range of measurements
232 (from 0.1% to 50%, 3 points/%). The correlation coefficient for the whole range was
233 $R^2=0.999$ ($p<0.001$) (fig. 1a) and $R^2= 0.974$ ($p<0.001$) for low values (from 0.1% to 1%) (fig.
234 1b). The CVs of reproducibility were $< 20\%$ for all chimerism percentages (fig. 2). The

235 sensitivity of this method was 0.1%, with a coverage of at least > 10,000 reads for each
236 marker. In our assays, no non-conforming quality criteria were observed.

237

238 Comparison of chimerism quantification

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

245

246 **Discussion:**

247 This study evaluated two new methods, an original combination of Crystal Digital PCR™
248 using GenDX® reagents and a ready-to-use NGS-based method using the “Devyser
249 Chimerism for NGS” kit to screen and quantify the chimerism.

250 Interestingly, Crystal Digital PCR™ is a system with 3-colour detection capability that
251 provides multiplexing power. We tested 11 triplex mixes of the GenDX® KMRtype kit to
252 discriminate informative donor/recipient pairs by qPCR. The three dyes of this kit are not
253 completely adapted to their use by Crystal Digital PCR™ because the ORG560 and RED610
254 fluorophores have wavelengths that are located at the end of the 2nd signal of the Naica
255 platform. Manual compensation was performed to obtain a more specific signal for these two
256 fluorophores. Thus, no assignment error, negative or positive, was found for these two
257 fluorophores in the other cases. The only two polymorphism identification errors involved
258 inexplicably two different probes associated with the FAM fluorophore. These probes had

259 been at least once positive and negative without error of identification for the other
260 genotyping. The design of these probes may not be completely suitable for cdPCR. More
261 genotyping is warranted to determine whether these errors are reproducible.

262 Informative markers of genotyping by NGS of each donor/recipient pair were automatically
263 identified by Advyser software. Each pair had at least three donor or recipient informative
264 markers identified. Manual changes, considering homozygous donor or recipient
265 polymorphisms only as informative, did not alter the quantification results of chimerism,
266 highlighting the right choice of automatic selection of informative polymorphisms.
267 Interestingly, the Devyser® panel comprises markers with a low bias from ethnic parameters,
268 allowing robust discrimination when analysing HLA identical siblings.

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

275 Thus, quantification of chimerism by cdPCR is a sensitive and accurate method (i.e., no
276 requirement for standard curves, efficiency controls and multiple replicates as qPCR) to
277 monitor HSCT patients. By performing all stages of digital PCR in a single consumable ready
278 to use, the Naica system offers a quick and easy solution. Moreover, this method is adapted to
279 clinical emergencies (results in < 4 h). Interestingly, the droplets in the chip can be stored for
280 up to 8 days at room temperature before reading. This advantage has made it possible to
281 perform runs during the night with a reading in the morning. The only technical limitation
282 identified was the phenomenon of electrocoalescence, leading to the fusion of droplets in the
283 chip. The use of an antistatic spray at each run controlled this effect.

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

289 The “Devyser for NGS” method reduces technical stages, manages informative markers,
290 reduces stockholding and reagent waste and performs the screening and monitoring of
291 chimerism in a single experiment. The number of quantification points depends on the flow
292 cell type and minimum coverage needed, varying from 11 quantification samples for MiSeq
293 v2 Micro (300 cycles) to 60 quantification samples for MiSeq v3 (150 cycles). Additionally,
294 evaluation of the associated software brings new perspectives regarding the flexibility of the
295 choice of informative markers and longitudinal follow-up of the patient, particularly
296 according to cell population. Furthermore, this technique can be automated and easily
297 integrated into the laboratory using NGS Illumina technology.

298 Finally, the correlation and concordance between the results obtained by the two methods
299 were very high, as revealed by regression and Bland–Altman analysis, indicating that these
300 two chimerism methods can be readily implemented in routine clinical diagnostics. Thus,
301 while NGS is designed for large series, cdPCR, by its flexibility of use (e.g., single
302 consumable ready to use, easy storage and results in <4 h), can be used in controls or
303 emergency. Additionally, chimerism analysis for both methods was performed with low
304 amounts of DNA, a common condition early after transplantation (usually until day +30)
305 when HSCT patients have a low white blood cell count.

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

308

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312

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

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351 **Figure Legends:**

352

353 Figure 1: Analytical performance of cdPCR and NGS against reference values. A series of
354 artificial chimerism mixtures, ranging between 0.05% and 50%, were quantified by cdPCR
355 and NGS and plotted against their reference values. (1a: whole range; 1b: low chimerism <
356 1%)

357 Figure 2: Reproducibility of the cdPCR and NGS methods. Each % chimerism was tested on
358 3 different experiments to calculate the CV of each method.

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

363 Figure 4: Concordance of the % chimerism results between cdPCR and ddPCR (4a) and
364 between NGS and ddPCR (4b) using the Bland–Altman plot. The Bland–Altman plot
365 includes the mean value (solid line) and 95% CI (dashed lines). A comparison of results
366 between cdPCR and ddPCR and between NGS and ddPCR revealed that all data points were
367 within the 95% CI.

368

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%	
	WB		90		0.3%	0.35%	0.3%	0.4%	
3	WB	ALL	140		0.1%	0.1%	0.1%	0.1%	
	BM		168		0.3%	0.3%	0.2%	0.3%	
	WB		15		60%	55%	55%	55%	
4	WB	IMMUNODEFICIENCY	30		50%	50%	50%	50%	55%
	WB		367		15%	17.5%	17%	15%	
	WB		1260		12%	14%	14%	12%	
	WB		1294		67%	64%	67.2%	60%	
5	WB	IMMUNODEFICIENCY	3128		60%	56%	60.7%	60%	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%	
	BM		90				45%	44%	
7	WB	ALL	147	relapse			15%		16%
	WB		153				12%	10.5%	
	CD3		153				0.9%	0.5%	
	WB		175				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%	

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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"> - Selection of donor/recipient informational markers - DNA and marker compartmentalisation in thousands of microreactors - Amplification of microreactors - Absolute quantification by digital reading (0 or 1) - Chimerism calculation by the informative marker/reference ratio 	<ul style="list-style-type: none"> - Multiplex PCR amplification of 24 informative markers (ins/del -75 bp), distributed on 17 autosomal chromosomes > one tube per sample - Preparation of an Illumina-compatible library: amplicon type > one tube per sample - Paired-end sequencing 2x75 bp - Automatic data analysis (genotyping and monitoring) and longitudinal follow-up of patient records
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	+	-

375 Table 2: Principle and technical characteristics of cdPCR and NGS

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

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KMR	Recipient 1		Donor 1		Recipient 2		Donor 2		Recipient 3		Donor 3		Recipient 4		Donor 4		
	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%

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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

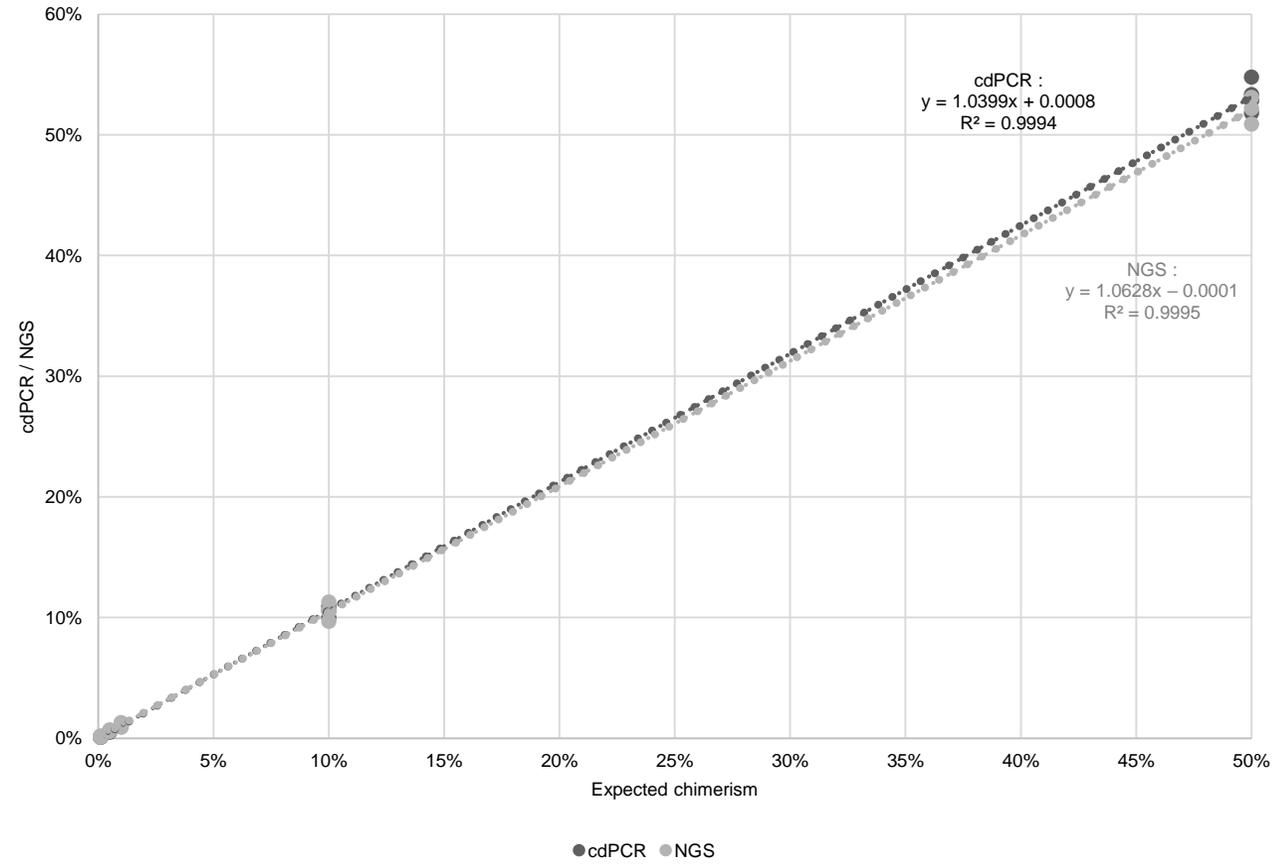


Figure 1b : low chimerism < 1%

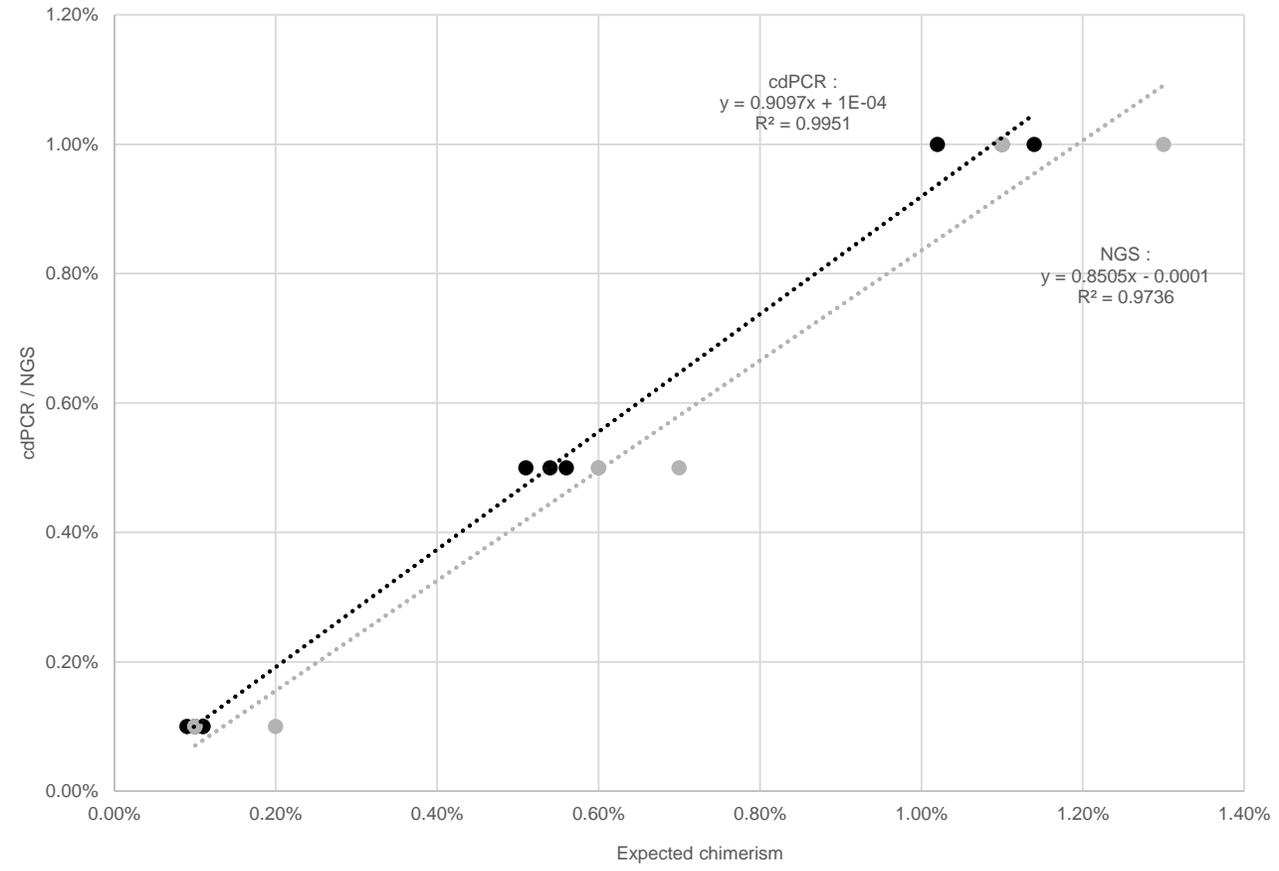


Figure 2: Reproducibility of cdPCR and NGS methods. Each % chimerism was tested on 3 different experiments to calculate the CV of each method.

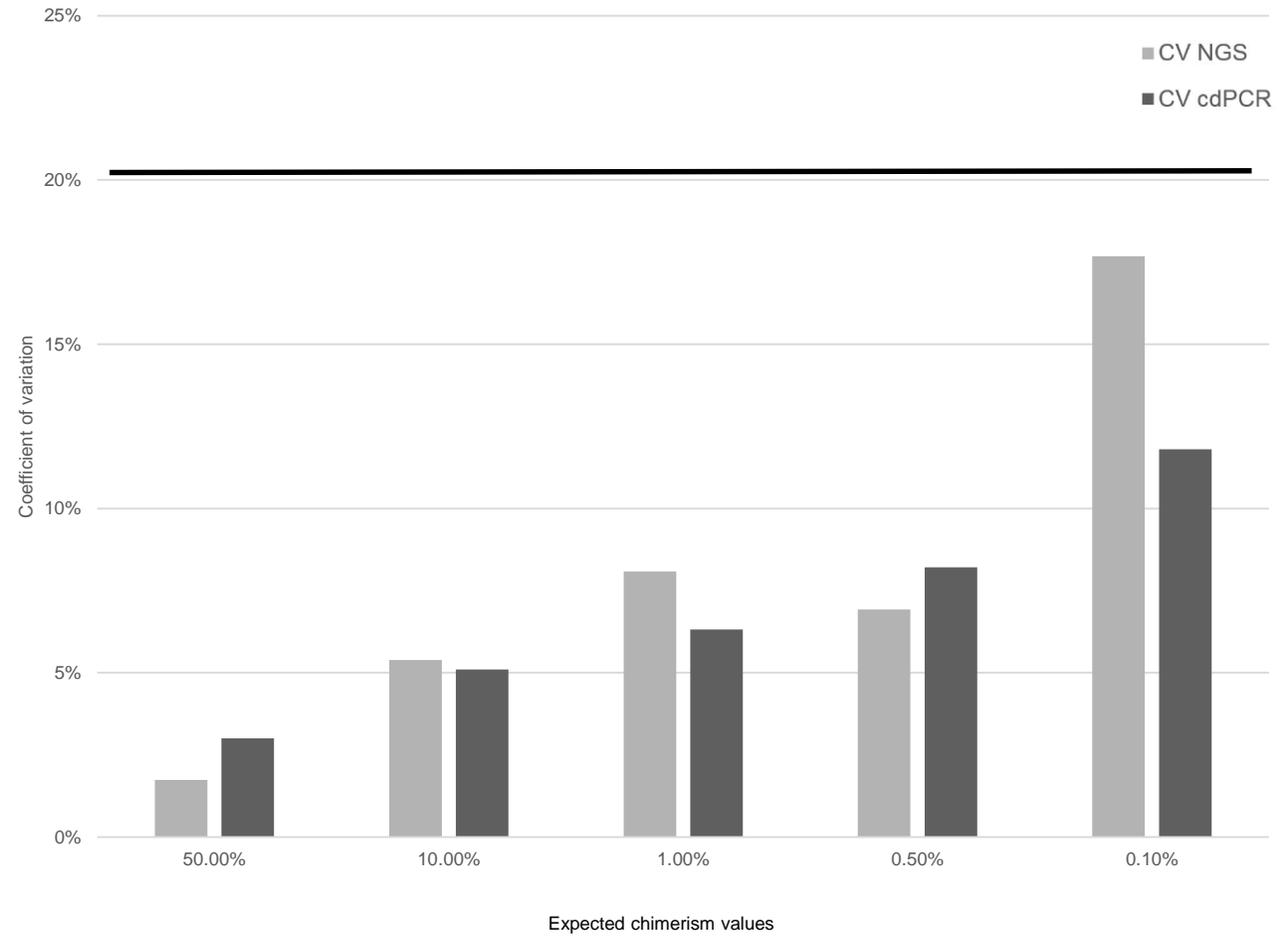


Figure 3a: Correlation of % chimerism results between cdPCR and ddPCR and between NGS and ddPCR, using regression curve.

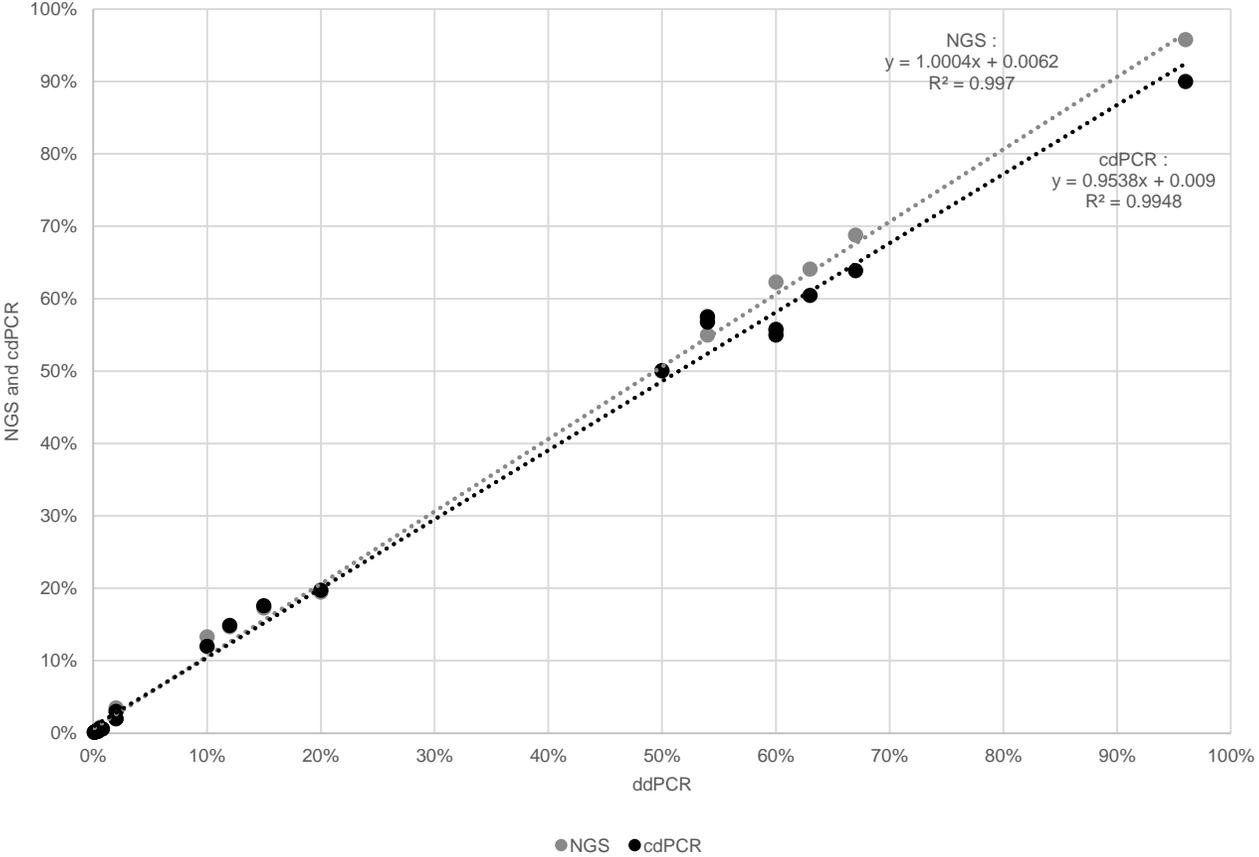


Figure 3b: Correlation of % chimerism results between NGS and qPCR / STR, using regression curve.

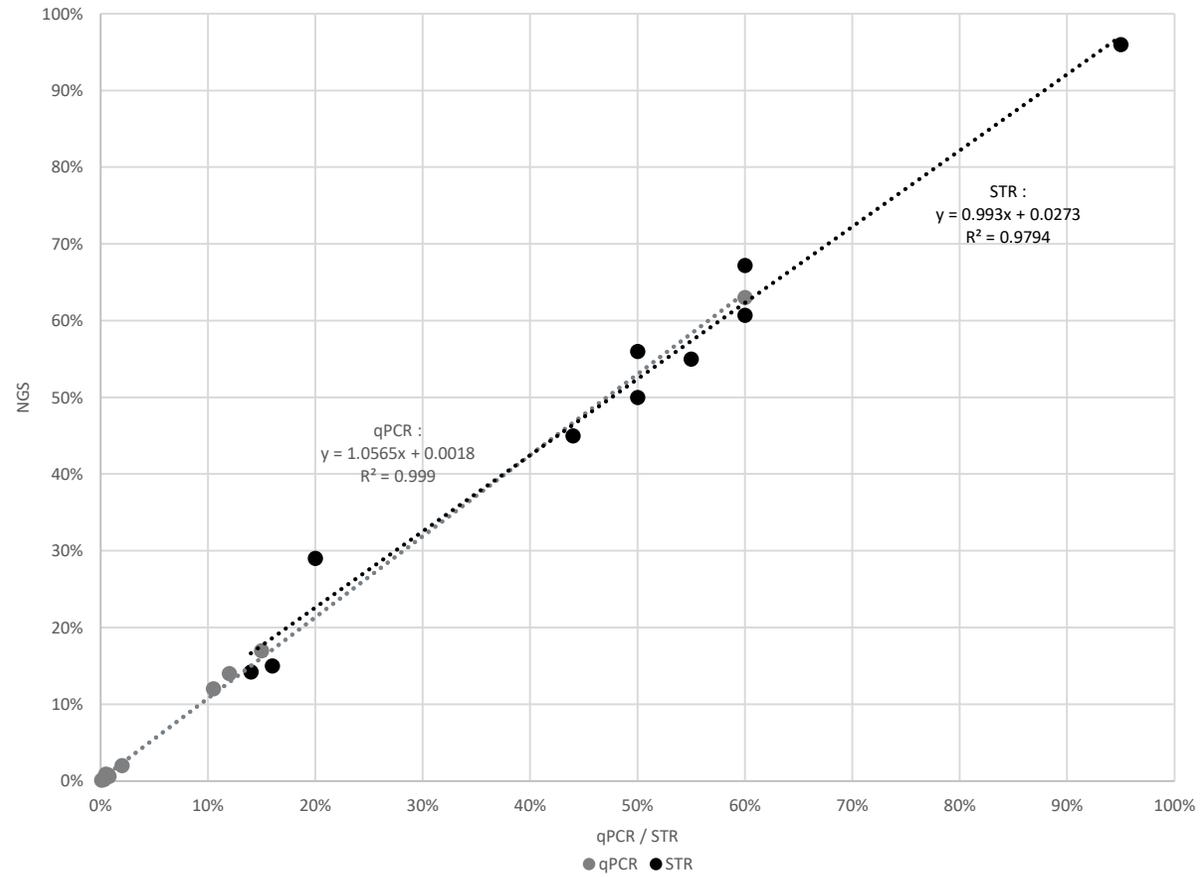


Figure 3c: Correlation of % chimerism results between cdPCR and qPCR / STR, using regression curve.

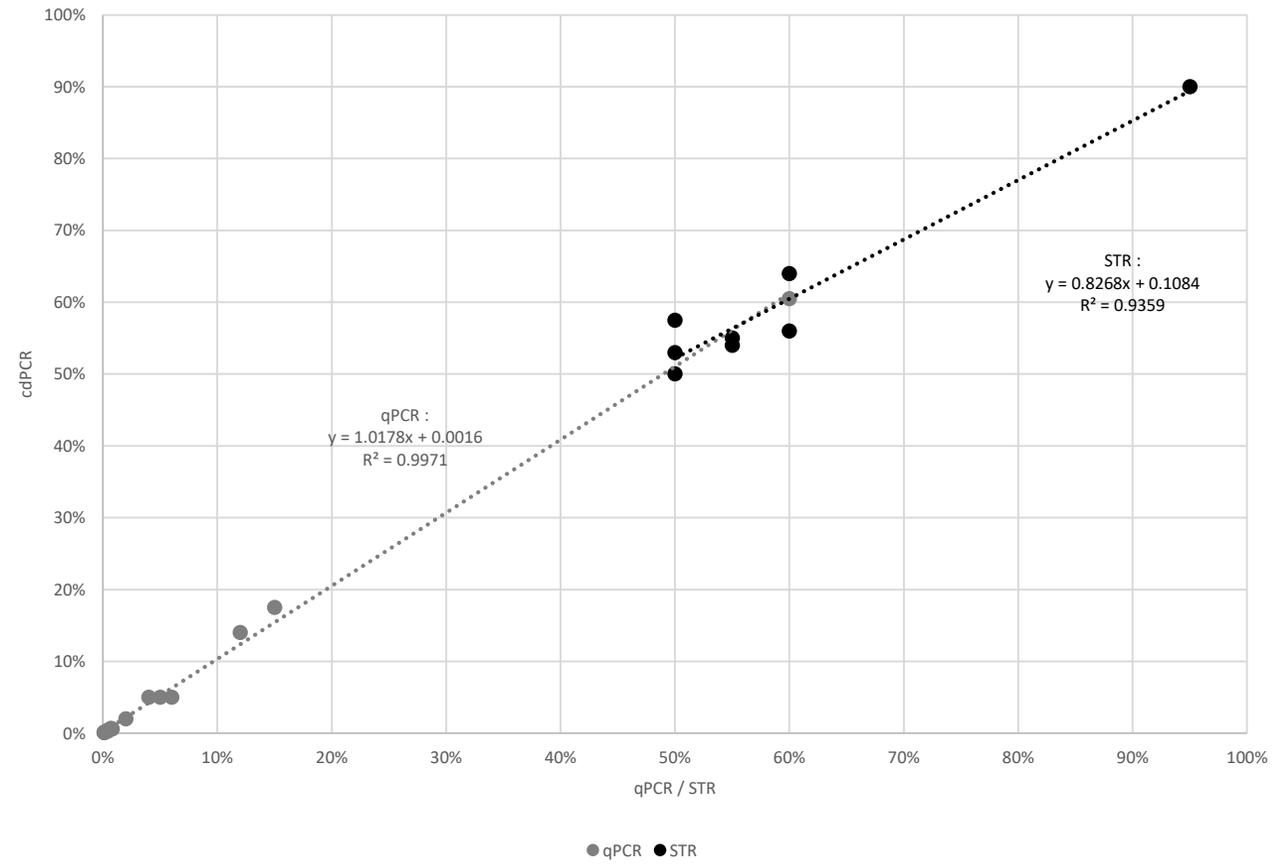


Figure 4: Concordance of % chimerism results between cdPCR and ddPCR (4a) using Bland-Altman diagram

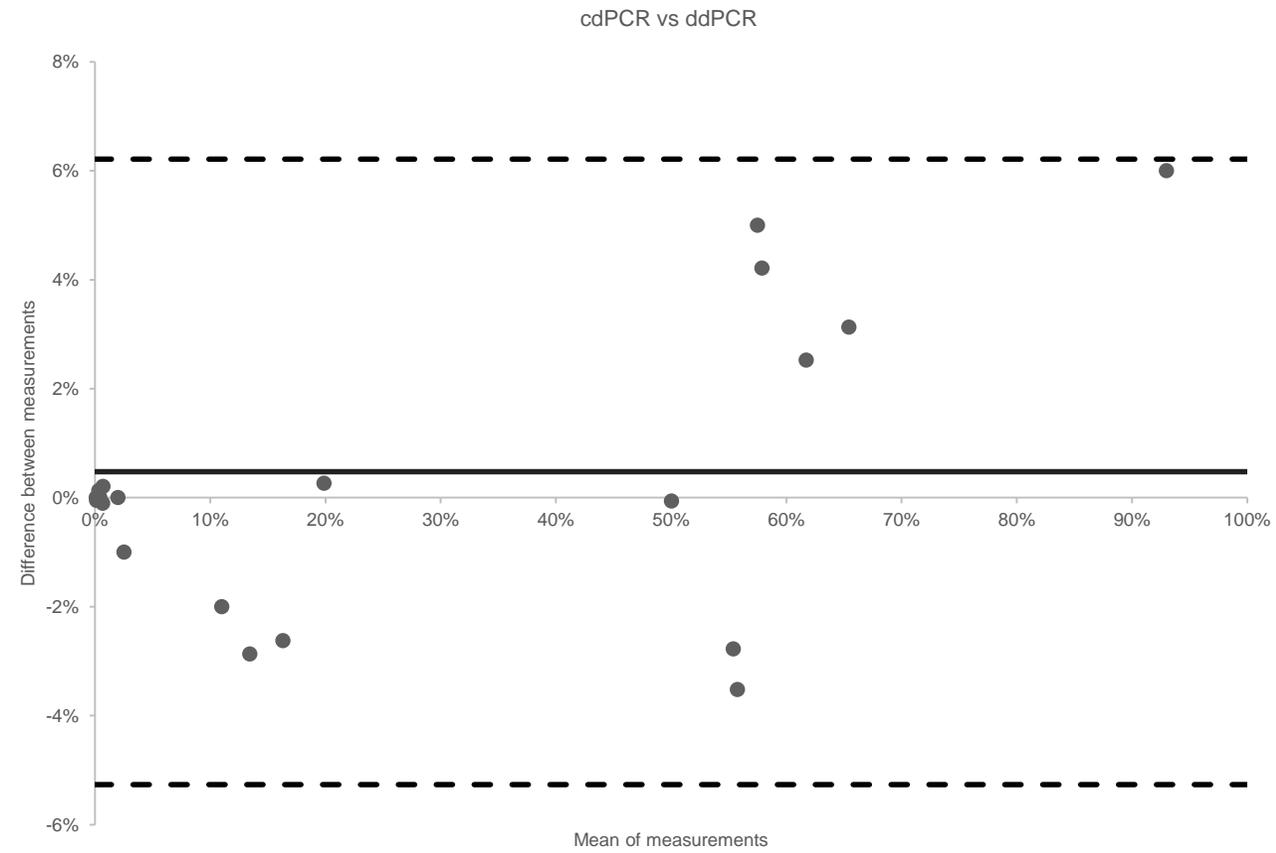


Figure 4: Concordance of % chimerism results between NGS and ddPCR (4b), using Bland-Altman diagram

