Journal of Cell Identity

Droplet digital PCR based analysis of chimerism using a panel of Indel markers

Victor Stefan Ionescu[1*#], Ioana Maria Lambrescu[1,2*], Gisela Gaina[1], Alexandra Popa[1,3], Dan Soare[1], Valeriu Bogdan Cismasiu[1#]

1 Victor Babes National Institute of Pathology, 050096 Bucharest, Romania
2 Department of Cellular and Molecular Biology and Histology, Carol Davila University of Medicine and Pharmacy, 050474 Bucharest, Romania
3 Department of Animal Production and Public Health, University of Agricultural Sciences and Veterinary Medicine, 050097 Bucharest, Romania
***** Contributed equally
Corresponding authors

Abstract

Cell identity, a fundamental concept in biology, emerges from the interplay of gene expressions, cellular functions, and genomic fingerprints. Chimerism refers to the presence of two genetically distinct types of cells within an individual. Monitoring of chimerism is an important tool to evaluate the evolution of engraftment after transplant and to detect the relapse of the underlying hematological neoplasm or the occurrence of graft rejection. Also, continuous monitoring is an essential tool that can provide guidance for early therapeutic intervention. Digital droplet PCR (ddPCR) is a promising alternative to the standard quantitative PCR to assess the chimerism below 1% due to an excellent performance in the assessment of small amounts of specific targets in a complex biological sample. Here, we describe the development of a panel of ddPCR based assays for chimeric analysis. We found a group of 25 indels showing a good discrimination power when tested with artificial mixtures of DNA samples.

Keywords:
droplet digital PCR, chimerism, indel polymorphism

DOI:
https://www.doi.org/xxxxxxxxxxjocixxxxxxxxx

*** Corresponding authors:**
victor.ionescu@ivb.ro,
valeriu.cismasiu@ivb.ro

Introduction

Cells show a remarkable diversity and specialization within the body. Their identities are shaped by intricate layers, including functional roles and genomic signatures. Functional identity emerges from gene expression patterns, protein interactions, and cellular responses. Genomic identity delves into somatic and epigenetic mutations at the DNA level. Mutations can alter cell identity by affecting gene expression, protein function, or signaling pathways. Specific indels may lead to the expression of unique cell surface markers, transcription factors, or other molecules that define cell types. Their impact on cell identity underscores their significance in shaping the intricate diversity of cells within an organism, and chimerism analysis helps us understand the dynamics of these mutated cell populations after transplantation.

The number and the type of cell fingerprints that need to be examined to achieve sufficient informative markers depend on the study goals. For instance, lineage specific markers enable the examination of gene functions in separate cell populations, like hematopoietic and non-hematopoietic cells. On the other hand, assessment of the post-transplant chimeras requires a more comprehensive set of markers to distinguish between cells with the same expression profile or function, but with different hereditary origins.

Chimerism analysis establishes the proportion between cell populations originating from two or more organisms, and it is widely used to monitor cell/tissue engraftment, graft rejection or disease relapse (Tozzo et al., 2021). Molecular assessment of chimerism relies on specific differences in the genomic DNA sequence (i.e., polymorphic markers) that distinguish donor from recipient cells. There are three types of genomic polymorphisms used in chimerism analysis: microsatellites (variable number tandem repeats, VNRT and short tandem repeats, STR), single nucleotide polymorphisms (SNPs) and insertion/deletions (InDels).

STR-polymerase chain reaction (PCR)-based genotyping is the predominant molecular method used for the detection of chimerism, since is fast, sensitive, reliable, and reproducible (Navarro-Bailón et al., 2020). However, STR analysis has a detection limit in the range of 1% to 5% and the accuracy at the lower limit of sensitivity is low (often, the coefficient of variation - CV - is above 25). The breakthrough in chimerism analysis came with the development of the quantitative real-time PCR (qPCR) and the TaqMan technology. A seminal study first showed that 0.1% of recipient DNA could be detected with high sensitivity in blood samples by qPCR of SNPs (Alizadeh et al., 2002). The procedure was further refined using InDel polymorphism, with a reporter sensitivity down to 0.01% (Kim et al., 2014; Valero-Garcia et al., 2019; Tyler et al., 2019). With this strategy, the risk of leukemia relapse is better predicted for patients having recipient chimerism below 5% (Jiménez-Velasco et al., 2005; Tyler et al., 2019).

The value of chimerism monitoring together with the standard tests for measurable residual disease (MRD) has been acknowledged lately for patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS), chronic myelomonocytic leukemia (CMML) and acute lymphocytic leukemia (ALL), after hematopoietic stem cell (HSC) transplant (Jacque et al., 2015; de Witte et al., 2017; Sellmann et al., 2018; Schuurhuis et al., 2018). Since the sensitivity of 0.1% or better is required for accurate MRD detection, the recommendation is to use qPCR and not STR to monitor autologous cell chimerism as an MRD marker (Schuurhuis et al., 2018).

Droplet digital PCR (ddPCR) is the latest generation of PCR, where standard end-point PCR and fluorescent PCR are combined. This technology has a slightly better sensitivity than qPCR, but it shows significantly greater accuracy and reduced variability over a broad range of target amounts. The partition of the target molecules into thousands of oil droplets, with each droplet holding one fluorescent PCR reaction due to the hydrophobic barrier, is the key feature of this method. The quantification of target molecule number in ddPCR is performed by counting single droplets as fluorescent positive or negative, which is much more reproducible than the Cq (Ct) values obtained by qPCR. This property makes ddPCR the preferred method for measuring tiny target fold-change among samples and detecting rare variants of targets. Therefore, ddPCR is currently at the forefront of a broad array of clinical applications, including chimerism analysis and monitoring cancer patients. Recent studies show that ddPCR allows

earlier detection of the rise in autologous chimerism in patients undergoing HSC transplant, when compared with STR or qPCR (Santurtún et al., 2017; Waterhouse et al., 2017; Mika et al., 2019; Valero-Garcia et al., 2019; Fortschegger et al., 2020).

Due to the high assay precision in the determination of microchimerism (below 0.01%), ddPCR is widely used for the detection of donor derived cell-free DNA (dd-cfDNA) as a marker for graft injury or rejection of transplanted kidneys, livers, hearts, and lungs (Schütz et al., 2017; Sigdel et al., 2018; Tanaka et al., 2018; Goh et al., 2019; Macher et al., 2019). Furthermore, two groups have shown that urine could be a reliable source of dd-cfDNA to track allograft rejection in kidney transplant using ddPCR (Lee et al., 2017; Kueng et al., 2023). The large variation of the urine dd-cfDNA amount among patients requires absolute quantification of the graft derived DNA, and this makes ddPCR a preferred method compared with the high-throughput sequencing (Kueng et al., 2023).

In this study we describe the selection of 25 InDels and the corresponding ddPCR assays to detect and quantify the mixt chimerism of targeted DNA.

Materials and methods

InDel selection

The InDels were selected from the public genetic variation database (dbSNP build 153). The polymorphisms were filtered through various criteria, including a minimal length difference of 15 bases between major and minor allele variants. Using C# language dynamic programming, the database was queried for variations of the insertion/deletion polymorphisms (DIPs) with a minor allele frequency (MAF) between 0.4 and 0.6 in European and American superpopulations from the 1000 Genomes project (all subpopulations included). Alleles with low complexity or repetitive structures were excluded, as well as the ones with other polymorphisms within flanking regions.

Assay design

For each selected InDel, we developed singleplex EvaGreen-based ddPCR assays guided by the allele-specific PCR principles. The reference assay was designed to quantify the total number of allelse containing the DIP marker. The assays were screened against the complete human genome through BLAST, to ensure the region was found in a single copy per haploid genome.

We designed primers with the annealing temperature (Ta) of 65C to ensure higher specificity. The melting temperature of both primers was adjusted to within 1°C difference. Temperature evaluations and primer-dimer formation were provided by OligoAnalyzer (Integrated DNA Technologies, Coralville, Iowa, USA). The amplicon length is less than 120 bp. Primers were ordered from Eurogentec (Liège, Belgium) and Integrated DNA Technologies (Coralville, Iowa, USA).

Sample preparation

Human blood samples from healthy volunteers were obtained in accordance with the Helsinki Declaration of 1975. The genomic DNA was extracted from white blood cells using the PureLink™ Genomic DNA Mini Kit (cat # K182001, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and assessed by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

The gDNA samples were genotyped, according to which potential combinations of samples were identified. Serial dilutions were made such that at least two different informative alleles could be evaluated from the same serial dilution. Considering the theoretical maximally attainable limit of quantification (LoQ), we made 2x serial dilutions, with 25 copies of informative allele as a lowermost quantity.

To minimize sample-related pipetting errors, the sample volume was set at 6 µL per reaction. This translates to a gDNA concentration of 1.78 ng/µL when considering the human genomic DNA size. Consequently, DNA LoBind® tubes (Eppendorf, Hamburg, Germany) were used to significantly reduce sample-to-surface binding in such dilute samples.

Reaction optimization

Reactions were assembled, including 10 µL of QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad, Hercules, California, USA) and 2 µL of each forward and reverse primer (stock 5uM), for a final 20 µL reaction volume. The partition of the reaction mix in oil was performed with the

QX200™ Droplet Generator (Bio-Rad, Hercules, California, USA). Thereafter, reactions were loaded in a 96-well plate, sealed, and amplified in the C1000 Touch Thermocycler (Bio-Rad, Hercules, California, USA). The droplet fluorescence quantification was performed with the QX200™ Droplet Reader and the QX Manager Software version 1.2 (Bio-Rad, Hercules, California, USA). In some cases, the fluorescence threshold was set up manually. Thus, the final proportions of simulated chimerism samples range from 50% to 0.78%.

For each allele-specific assay, features such as Ta, number of cycles and primer concentration were optimized to minimize nonspecific or ambiguous amplification known as "ddPCR rain". Optimal values for these parameters can be found in **Supplementary Table 1**. Robustness

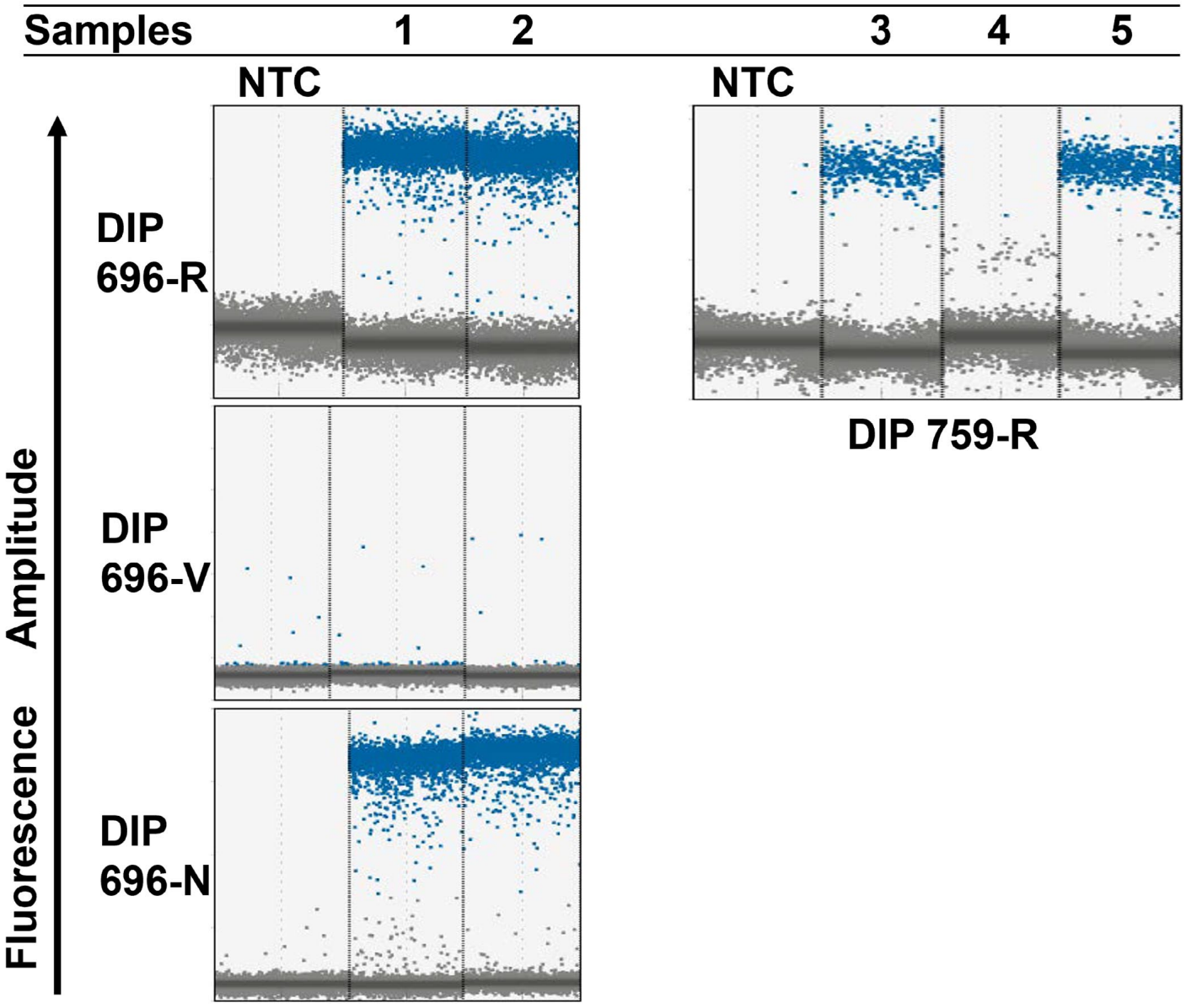

Figure 1.
Genotyping with ddPCR.

First column: Reaction specificity. Samples 1 and 2 are homozygous for the DIP 696-R (reference) allele. Signal comparable to NTC is observed for the DIP 696-V (variant) allele. The reference assay DIP 696-N demonstrates a droplet distribution like 696-R indicating the genotype R/R for samples 1 and 2.

Second column: Relevance of different genotypes as positive controls. Samples 3 and 5, in which allele 759 R was detected, display a clear band of fluorescent droplets as compared to a different pattern of amplification in sample 4, hence deemed nonspecific.

NTC No template control.

was ensured by performing PCRs on different days by different experimenters.

To determine the most appropriate reference tests, we loaded reactions with the same amount of gDNA and compared which results consistently yielded similar copy numbers across replicates. In the first approach, we generated confidence intervals of different levels (38%, 50%, 68%) and chose the assays that fell within those levels. In the second approach, we employed a sum of squared errors method and selected the assays showing the best fit.

Statistical interpretation

We employed the R programming language, version 4.0.1, and Microsoft Excel 2021 for all statistical analyses. For each assay, we performed a correlation analysis of the simulated chimerism versus experimental results using Pearson's correlation coefficient. Data was considered statistically significant for $p < 0.05$ and $R^2 > 0.98$.

Results and discussion

The screening and characterization of cell fingerprints benefit greatly from PCR-based methods considering their low cost, high accuracy, and the fact that they often provide multidimensional read-outs. As a result, they assist in monitoring a variety of malignancies and other pathologies by supplying scientists with vital data on cell identity. Mutations could modify the identity of a cell by impacting gene expression, the function of proteins, or the routes of signaling. It is still necessary to determine the most sensitive methods of disease diagnosis and monitoring. Furthermore, these technologies can provide a fresh perspective on the extensive realm of cellular identity, including both normal and abnormal ones.

The SNPs are one variable base while the InDel variants differ by several consecutive bases, making the latter more suitable for a primer-target annealing with high specificity and the reduction of false positive reactions. The development of high-throughput sequencing has led to a rapid increase in the number of genetic variations identified in the human genome.

The InDels were selected by analysis of the public genetic variation database (dbSNP build 153) with the following starting criteria: a minimal length difference of 15 bases between major and minor allele variants; the minor allele frequency (MAF) bigger than 0.4 in the case of European populations. The ideal informative marker should be homozygous: both alleles as major variant in one genome and both alleles as minor variant in the other genome. Therefore, we set up the minimal cutoff of homozygote frequency at 0.25 (any variant).

Table 1 shows the allele and genotype frequencies for the DIPs selected in this study, as well as the chromosome location.

For each DIP marker we designed primers specifically for detection and quantification of the major and minor alleles. In some cases, we also designed primers to quantify the DIP locus regardless of the allele variant. The primers were validated by qPCR using synthetic DNA targets (data not shown). By adjustment of several ddPCR parameters, we established uniform assay conditions to run PCR reactions simultaneously for different DIP markers.

Figure 1 shows representative ddPCR results: each spot represents one oil droplet; the vertical distribution of the drops marks the fluorescence intensity of the EvaGreen dye. Most of the PCR positive spots are distributed in a narrow upper area (blue spots), because reactions reach the fluorescence plateau.

To explore the effectiveness of the DIP for chimerism analysis, we genotyped the blood DNA samples collected from healthy volunteers. The allele specific PCR assays were tested for 5 DIP markers by qPCR and most of the results were confirmed with ddPCR. The data summary is presented in **Table 2**.

The data indicates that there is at least one informative marker for 96% of possible mixed chimeras and two or more markers for 63% of total combinations. Remarkably, more than 50% of the genotypes are homozygous.

We assessed the sensitivity and linearity of the assays with a simulated chimerism made by mixing the DNA of two individuals. Serial dilutions were prepared as described in Material and Methods.

Figure 2 shows the results obtained with four DIP markers. The data indicates a good correlation between the measured and expected degrees of chimerism ($R > 0.98$).

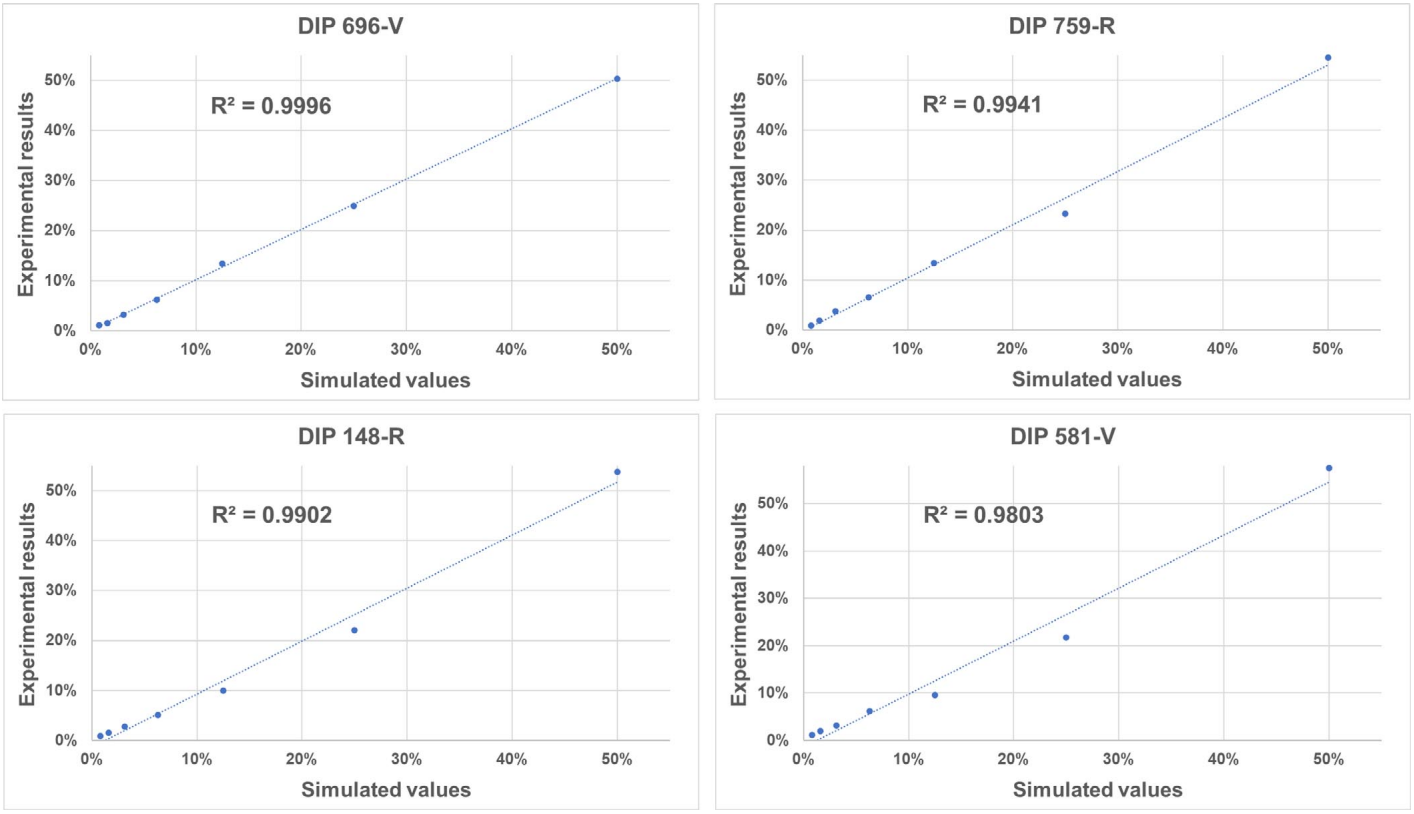

Figure 2.
Linear regression analysis of simulated and experimental chimerism values in four assays.
The dynamic range of the ddPCR system shows a linear response from 50% down to values of less than 1%, in full agreement with the expected linear model (Pearson's correlation coefficient r › 0.99, p ‹ 0.05 for all 4 assays).

Next, we tested the technical accuracy of the ddPCR assays, when simulated chimerism is below 10%.

As shown in **Table 3**, the coefficient of variation was smaller than 25%. Although the coefficient of variation of the ddPCR amplifications was relatively low, we have noticed that the pipetting errors significantly impact the data quality. The pipetting precision depends on several factors, including pipette calibration and the quality of the tip plastics. Therefore, the reference ddPCR assays were run in parallel with DIP markers to determine how many copies of the human genome are present in each sample.

In conclusion, we have developed a sensitive assay with 25 DIP markers to assess chimerism with the ddPCR platform. The number of markers in an assay impacts the percentage of chimerism and consequently determines the precision of the assay. The chimerism could be monitored with high sensitivity by both qPCR and ddPCR methods. However, it is likely that ddPCR will be over the edges of qPCR regarding the reproducibility performance, because sample partitioning is far more precise than the Cq (Ct) output of the qPCR. This feature might be essential during the longitudinal monitoring of a patient chimerism, for early disease relapse or graft rejection prediction. Overall, owing to their advantages, such assays have the potential to become cost-effective guides for medical decisions in certain diseases where indels play a clear part in the pathological change of cell identity.

Funding

This work was supported by UEFISCDI, Ministry of Education and Scientific Research, Romania, under grant PN-III-P2-2.1-PED-2016-1932 (VBC), and by Ministry of Education and Scientific Research, Romania, under grant PN19.29.01.03 (VBC).

Competing interests/conflict of interests

The authors are inventors of one pending patent application for the use of the selected DIP markers (DNA oligos) in ddPCR and qPCR based genotyping and quantification related to any type of chimerism or forensic analysis.

References

Alizadeh M, Bernard M, Danic B, et al. (2002) Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. Blood. 99(12):4618-4625. doi:10.1182/blood.v99.12.4618

Fortschegger M, Preuner S, Printz D, et al. (2020) Detection and monitoring of lineage-specific chimerism by digital droplet PCR-based testing of deletion/insertion polymorphisms. Biol Blood Marrow Transplant. 26(6):1218-1224. doi:10.1016/j.bbmt.2020.02.016

Goh SK, Do H, Testro A, et al. (2019) The Measurement of donor-specific cell-free DNA identifies recipients with biopsy-proven acute rejection requiring treatment after liver transplantation. Transplant Direct. 5(7):e462. doi:10.1097/TXD.0000000000000902

Jacque N, Nguyen S, Golmard JL, et al. (2015) Chimerism analysis in peripheral blood using indel quantitative real-time PCR is a useful tool to predict post-transplant relapse in acute leukemia. Bone Marrow Transplant. 50(2):259-265. doi:10.1038/bmt.2014.254

Jiménez-Velasco A, Barrios M, Román-Gómez J, et al. (2005) Reliable quantification of hematopoietic chimerism after allogeneic transplantation for acute leukemia using amplification by real-time PCR of null alleles and insertion/deletion polymorphisms. Leukemia. 19(3):336-343. doi:10.1038/sj.leu.2403622

Kim SY, Jeong MH, Park N, et al. (2014) Chimerism monitoring after allogeneic hematopoietic stem cell transplantation using quantitative real-time PCR of biallelic insertion/deletion polymorphisms. J Mol Diagn. 16(6):679-688. doi:10.1016/j.jmoldx.2014.06.005

Kueng N, Arcioni S, Sandberg F, et al. (2023) Comparison of methods for donor-derived cell-free DNA quantification in plasma and urine from solid organ transplant recipients. Front Genet. 14:1089830. doi:10.3389/fgene.2023.1089830

Lee H, Park YM, We YM, et al. (2017) Evaluation of Digital PCR as a Technique for Monitoring Acute Rejection in Kidney Transplantation. Genomics Inform. 15(1):2-10. doi:10.5808/GI.2017.15.1.2

Macher HC, García-Fernández N, Adsuar-Gómez A, et al. (2019) Donor-specific circulating cell free DNA as a noninvasive biomarker of graft injury in heart transplantation. Clin Chim Acta. 495:590-597. doi:10.1016/j.cca.2019.06.004

Mika T, Baraniskin A, Ladigan S, et al. (2019) Digital droplet PCR-based chimerism analysis for monitoring of hematopoietic engraftment after allogeneic stem cell transplantation. Int J Lab Hematol. 41(5):615-621. doi:10.1111/ijlh.13073

Navarro-Bailón A, Carbonell D, Escudero A, et al. (2020) Short tandem repeats (STRs) as biomarkers for the quantitative follow-up of chimerism after stem cell transplantation: Methodological Considerations and Clinical Application. Genes (Basel). 11(9):993. doi:10.3390/genes11090993

Santurtún A, Riancho JA, Arozamena J, et al. (2017) Indel analysis by droplet digital PCR: a sensitive method for DNA mixture detection and chimerism analysis. Int J Legal Med. 131(1):67-72. doi:10.1007/s00414-016-1422-4

Sellmann L, Rabe K, Bünting I, et al. (2018) Diagnostic value of highly-sensitive chimerism analysis after allogeneic stem cell transplantation. Bone Marrow Transplant. 53(11):1457-1465. doi:10.1038/s41409-018-0176-7

Schütz E, Fischer A, Beck J, et al. (2017) Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: A prospective, observational, multicenter cohort study. PLoS Med. 14(4):e1002286. doi:10.1371/journal.pmed.1002286

Schuurhuis GJ, Heuser M, Freeman S, et al. (2017) Minimal/measurable residual disease in AML: a consensus document from the European LeukemiaNet MRD Working Party. Blood. 131(12):1275-1291. doi:10.1182/blood-2017-09-801498

Sigdel TK, Archila FA, Constantin T, et al. (2018) Optimizing detection of kidney transplant injury by assessment of donor-derived cell-free DNA via massively multiplex PCR. J Clin Med. 2018;8(1):19. doi:10.3390/jcm8010019

Tanaka S, Sugimoto S, Kurosaki T, et al. (2018) Donor-derived cell-free DNA is associated with acute rejection and decreased oxygenation in primary graft dysfunction after living donor-lobar lung transplantation. Sci Rep. 8(1):15366. doi:10.1038/s41598-018-33848-3

Tozzo P, Delicati A, Zambello R, Caenazzo L. (2021) Chimerism monitoring techniques after hematopoietic stem cell transplantation: an overview of the last 15 years of innovations. Diagnostics (Basel). 11(4):621. doi:10.3390/diagnostics11040621

Tyler J, Kumer L, Fisher C, et al. (2019) Personalized chimerism test that uses selection of short tandem repeat or quantitative PCR depending on patient's chimerism status. J Mol Diagn. 21(3):483-490. doi:10.1016/j.jmoldx.2019.01.007

Valero-Garcia J, González-Espinosa MDC, Barrios M, et al. (2019) Earlier relapse detection after allogeneic haematopoietic stem cell transplantation by chimerism assays: Digital PCR versus quantitative real-time PCR of insertion/deletion polymorphisms [published correction appears in PLoS One. 14(3):e0213966]. PLoS One. 2019;14(2):e0212708. doi:10.1371/journal.pone.0212708

Waterhouse M, Pfeifer D, Follo M, et al. (2017) Early mixed hematopoietic chimerism detection by digital droplet PCR in patients undergoing gender-mismatched hematopoietic stem cell transplantation. Clin Chem Lab Med. 55(8):1115-1121. doi:10.1515/cclm-2016-0900

de Witte T, Bowen D, Robin M, et al. (2017) Allogeneic hematopoietic stem cell transplantation for MDS and CMML: recommendations from an international expert panel. Blood. 129(13):1753-1762. doi:10.1182/blood-2016-06-724500

DIP code	Chromosome	Variant	MAF (EUR)	R/R (EUR)	V/V (EUR)	R/V (EUR)	MAF (AMR)	R/R (AMR)	V/V (AMR)	R/V (AMR)
151	1	indel	0.5716	0.2410	0.3840	0.3760	0.5288	0.2910	0.3490	0.3600
905	1	indel	0.4720	0.2840	0.2290	0.4870	0.454	0.2420	0.3340	0.4240
359	2	indel	0.4423	0.3240	0.2090	0.4670	0.4654	0.2650	0.1960	0.5390
148	2	indel	0.5060	0.2520	0.2640	0.4830	0.5922	0.1820	0.3660	0.4520
696	3	deletion	0.5080	0.2600	0.2760	0.4630	0.5	0.2450	0.2450	0.5100
716	3	deletion	0.5835	0.1770	0.3440	0.4790	0.4366	0.3340	0.2070	0.4580
389	5	indel	0.4155	0.3280	0.1590	0.5130	0.4121	0.3260	0.1500	0.5240
781	7	deletion	0.5984	0.1690	0.3660	0.4650	0.4481	0.3110	0.2070	0.4810
122	8	indel	0.4523	0.3100	0.2150	0.4750	0.5836	0.2050	0.3720	0.4240
145	8	indel	0.5835	0.1670	0.3340	0.4990	0.4899	0.2680	0.2480	0.4840
156	9	indel	0.5179	0.2620	0.2960	0.4390	0.5476	0.2560	0.3520	0.3920
437	9	indel	0.4520	0.3120	0.2170	0.4710	0.345	0.4520	0.1440	0.4030
946	10	insertion	0.4125	0.3540	0.1790	0.5150	0.5389	0.2070	0.2850	0.5070
829	10	indel	0.4911	0.2660	0.2490	0.4850	0.4078	0.3630	0.1790	0.4580
920	11	deletion	0.5646	0.2010	0.3300	0.4690	0.5014	0.2420	0.2450	0.5130
423	13	indel	0.4270	0.3420	0.1970	0.4610	0.258	0.5590	0.0750	0.3660
804	13	indel	0.4284	0.3540	0.2110	0.4350	0.4914	0.2480	0.2310	0.5220
198	13	deletion	0.5706	0.2050	0.3460	0.4490	0.4827	0.2850	0.2510	0.4640
881	13	deletion	0.5845	0.1800	0.3500	0.4690	0.4063	0.3600	0.1730	0.4670
581	17	insertion	0.4096	0.3540	0.1730	0.4730	0.4107	0.3340	0.1560	0.5100
354	18	indel	0.4056	0.3580	0.1690	0.4950	0.4568	0.2820	0.1960	0.5220
759	18	deletion	0.5616	0.2820	0.4060	0.3120	0.4856	0.3920	0.3630	0.2450
844	22	indel	0.4046	0.3660	0.1750	0.4590	0.5375	0.2190	0.2940	0.4870
402	22	indel	0.5517	0.2190	0.3220	0.4590	0.464	0.2940	0.2220	0.4840
248	X	indel	0.5091	0.121 (R/R) / 0.231 (R/0)	0.127 (V/V) / 0.247 (V/0)	0.2740	0.5687	0.086 (R/R) / 0.225 (R/0)	0.17 (V/V) / 0.265 (V/0)	0.2540

Table 1
Characteristics of the Indels included in this study.
MAF minor allele frequency;
R major allele variant (reference allele);
V minor allele variant (variant allele);
EUR European populations;
AMR American populations;
"R/o" and "V/o" indicate male samples.

DIP code	Analysis		Genomic Frequency			Samples							
	qPCR	ddPCR	R/R	R/V	V/V	1	2	3	4	5	6	7	8
156	✓	✓	0.63	0.12	0.25	V/V	V/V	R/R	R/R	R/R	R/V	R/R	R/R
354	✓	✓	0	0.5	0.5	V/V	R/V	R/V	R/V	V/V	V/V	R/V	V/V
696	✓	ND	0.29	0.42	0.29	R/R	ND	R/R	V/V	R/V	V/V	R/V	R/V
759	✓	✓	0.12	0.5	0.38	R/V	R/R	V/V	R/V	V/V	R/V	V/V	R/V
844	✓	✓	0	0.62	0.38	R/V	V/V	R/V	R/V	R/V	V/V	R/V	V/V

Table 2
Summary of the discriminative test

ND not determined;
R major allele variant (reference allele);
V minor allele variant (variant allele).

Analytical results: DIP 148-R

Serial dilution	Experimental Chimerism	Number of target molecules					Coefficient of variation
		Experiment 1	Experiment 2	Experiment 3	Average	Standard deviation	
NTC	0%	2	1	0	1	1	
SD1	9.8%	229	218	215	221	7	3.4%
SD2	6.0%	124	145	132	134	10	7.7%
SD3	2.6%	54	55	81	63	15	23.8%
SD4	1.6%	36	32	40	36	4	11.1%
SD5	0.8%	21	17	19	19	2	9.7%

Analytical results: DIP 581-V

Serial dilution	Experimental Chimerism	Number of target molecules					Coefficient of variation
		Experiment 1	Experiment 2	Experiment 3	Average	Standard deviation	
NTC	0%	0	2	4	2	2	
SD1	9.7%	234	243	177	218	36	16.3%
SD2	6.3%	151	132	143	142	10	6.7%
SD3	3.1%	88	77	64	76	12	16.0%
SD4	1.9%	44	34	54	44	10	23.0%
SD5	1.2%	31	23	28	27	4	14.8%

Table 3
Comparison of two DIP markers for quantification accuracy

Assay	Primer 1	Primer 2	T_a (°C)	Amplification cycles	Primer concentration (nM)
148-R	148-REF-F	148-COM-R	62	40	500-900
156-R	156-REF-R	156-COM-F-2	63	35	500
354-D	354-R	354-COM-F	61.2	35	500
354-V	354-VAR-R-2	354-COM-F	61.4	35	500
359-D	359-R	359-COM-F	62.4	35 or 36	500
581-V	581-VAR-R	581-COM-F	62	40	500-900
696-R	696-REF-R	696-COM-F	62.6	35	500
696-V	696-VAR-R	696-COM-F	61.5 - 61.6	35 or 36	500
759-R	759-REF-F	759-COM-R-2	62.5 - 62.7	35 or 36	500
946-D	946-R	946-COM-F-2	62.2	35-40	500-900

Supplemental Table 1.
Illustrative parameters of optimized ddPCR assays
Ta annealing temperature;
nM nanomolar concentration.