

Multiplex droplet digital PCR for 22q11.2 microdeletions screening and DiGeorge syndrome diagnostics

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ABSTRACT

Background and aims: DiGeorge syndrome (DGS) is a genetic disorder manifesting in polymorphic symptoms related to developmental abnormalities of various organs including thymus. DGS is caused by microdeletions in the 22q11.2 region between several low copy repeats (LCR) occurring in approximately 1 in 4000 live births. Diagnosis of DGS relies on phenotypic examination, qPCR, ultrasound, FISH, MLPA and NGS which can be relatively inaccurate, time-consuming, and costly.

Materials and methods: A novel multiplex droplet digital PCR (ddPCR) assay was designed, optimized and validated for detection and mapping 22q11.2 microdeletions by simultaneous amplification of three targets — *TUPLE1*, *ZNF74*, *D22S936* — within the deletion areas and one reference target — *RPP30* — as an internal control.

Results: The assay reliably identified microdeletions when the template concentration was >32 copies per reaction and successfully detected LCR22A-B, LCR22A-C, LCR22A-D, and LCR22B-C deletions in clinical samples from 153 patients with signs of immunodeficiency. In patients with the microdeletions, flow cytometry detected a significant increase in B-cell and natural killer cell counts and percentages, while T-cell percentages and T-cell receptor excision circle (TREC) numbers decreased.

Conclusion: The designed ddPCR assay is suitable for diagnosing DGS using whole blood and blood spots.

1. Introduction

DiGeorge syndrome (DGS) is a complex genetic disorder characterized by a combination of symptoms caused by microdeletions in the 22q11.2 region of the long arm of chromosome 22 [1]. These microdeletions lead to anomalies in the pharyngeal arches during embryogenesis, resulting in a broad spectrum of features. The most common symptoms of DGS include cardiovascular abnormalities, hypoparathyroidism, hypocalcemia, and thymus hypoplasia. Other clinical manifestations can include developmental delays, renal, palatal, gastrointestinal, neurological, and ophthalmological anomalies [2]. This highly diverse phenotypic representation initially led to the description

of several congenital disorders — such as Shprintzen-Goldberg syndrome, Cayler cardiofacial syndrome, velocardiofacial syndrome, and Sedlyakova syndrome — which were later recognized as synonyms for the same congenital condition [3]. The severity of DGS also varies greatly, from individuals who are almost healthy and unaware of their condition to subjects who could die during the first year of life without hematopoietic or thymic transplantation.

Among various other features of DGS, cardiac abnormalities and thymus hypoplasia can be life-threatening symptoms. While the degree of thymus anomalies varies, a certain group of DGS patients suffer from a complete lack of this organ. These individuals develop severe combined immunodeficiency (SCID), which can be lethal if left untreated [4].

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Other subjects with milder DGS symptoms may have a higher risk of recurrent and opportunistic infections, as well as autoimmune reactions. Cardiac symptoms are the most common in DGS, affecting nearly 80 % of patients, and are also the main cause of lethal outcomes [5]. Thus, prenatal or neonatal diagnostics are crucial for early intervention and reducing the mortality rate.

Microdeletions in the 22q11.2 locus are mostly of de novo origin and are hemizygous [6]. The estimated frequency of 22q11.2 deletions in Europe is 1 in 4000 live births [7,8]. The actual presence of these deletions may be higher, as not all individuals with 22q11.2 deletions develop DGS symptoms and undergo testing for DGS. Hindering phenotypic screening, the origin is linked with the prevalence of craniofacial abnormalities [9]. Afro-American infants with DGS may not exhibit the characteristic dysmorphic features known in children from other groups [10,11]. Many populations have not been studied regarding DGS frequency, necessitating further broader studies. Notably, 22q11.2 deletions are the most frequent among the human genome and occur in approximately 1 per 1000 fetuses [12,13]. The observed discrepancy can be explained by underestimation of subjects with DGS or embryonic lethality.

Homologous recombination between low copy repeats (LCRs) is believed to be the cause of 22q11.2 deletions. LCRs in the 22q11.2 locus are located relatively close to each other, which appears to facilitate unusually high frequencies of de novo recombination events [14]. The distance between LCR22A and LCR22D is approximately 3 Mbp, and this deletion is the most common among patients with DGS [15]. Other nested deletions, such as LCR22A-B, LCR22A-C, and LCR22B-D, are less prevalent [16]. Additionally, other deletions involving distal LCRs have been found and can result in different phenotypes [17]. The deleted region encompasses around 90 genes with various functions. Among these genes, T-box transcription factor 1 (*TBX1*) plays a role in pharyngeal arches development. In mouse models, *Tbx1* heterozygous deletions have led to pharyngeal, cardiac, parathyroid, and thymic embryonic anomalies, which are similar to characteristic DGS features [18–22]. It should be noted that the microdeletions alone cannot solely account for the highly polymorphic DGS symptoms, and other factors participate in the pathogenesis of the disease.

Diagnosis of DGS relies on several approaches, including physical examination, echocardiogram, ultrasound, blood tests, and molecular techniques [3]. Given the broad spectrum of DGS symptoms and their varying severity, classical craniofacial screening can be problematic, making laboratory tests necessary for a differential diagnosis of DGS. Echocardiogram and ultrasound can help detect cardiac, thymic, and other anomalies in fetuses and newborns, although they may still be subjective. Molecular methods, such as FISH, multiplex ligation-dependent probe amplification (MLPA), comparative genomic hybridization (CGH), NGS, quantitative PCR (qPCR), and digital PCR (dPCR), can directly identify 22q11.2 deletions using blood spots, whole blood, or fetal cell-free DNA for prenatal screening [23–29]. However, these techniques have limitations, including analytical sensitivity, the need for a high amount of DNA for analysis, high cost per test, and high demands for personnel and equipment. The presence of microdeletions also does not allow for the prediction of the exact phenotypic pattern of DGS. Therefore, a complex approach involving multiple tests is necessary for robust diagnostics and prognosis determination.

Digital PCR (dPCR) is a powerful method for reference-independent absolute quantification of nucleic acids, estimation of copy number, and detection of rare structural polymorphisms. While the dynamic range of digital PCR platforms is generally lower than that of qPCR, dPCR offers increased precision by directly counting DNA or RNA molecules without relying on standard samples quantified by independent methods [30]. Several dPCR tests have been proposed for DGS screening, based on the quantification of multiple loci within the region affected by 22q11.2 deletions [28,29]. However, these assays have been monoplex or duplex, which increases the cost per analysis due to the need for separate reactions to analyze multiple loci for each patient. Here, we present a

multiplex ddPCR test designed to map classical deletions. The test was validated using clinical samples from 153 patients with primary immunodeficiency, and the obtained results regarding deletion types were compared with clinical signs.

2. Material and methods

2.1. Samples and DNA extraction

Blood samples were obtained from patients who were treated at G. Speransky Children's Hospital No. 9 in Moscow. Informed consent was obtained from all subjects involved in the study. The study was performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The study's protocol was approved by the ethics committee at G. Speransky Children's Hospital No. 9 (NO. 1, 21 January 2014). Informed consent was obtained from all subjects involved in the study. DNA was extracted from blood samples using the Ribo-prep (Amplisens®, Russia) following the manufacturer's protocol. The purified DNA was quantified using NanoDrop Lite (Thermo Fisher Scientific, USA) and qPCR. Clinical samples were collected for routine examination of children admitted to the Immunopathology Department. PCR TREC/KREC assessment and flow cytometry were performed in parallel from the same EDTA blood tube using a commercially available kit IMMUNO-BIT (ABV-test, Russia) [31].

2.2. Flow cytometry

Three-Four colour flow cytometric immunophenotyping with directly labeled monoclonal antibodies was used to determine the following immune cell subsets: CD3- CD19+, CD3-CD(16 + 56)+, CD3+ CD4+ and CD3+ CD8+ following manufacturer's protocol. In brief, 50 µl aliquots of blood were incubated for 15 min in the dark at room temperature with combinations of optimally titrated fluorochrome-conjugated monoclonal antibodies: CD45 PE-Cy5/CD3 FITC/CD19 PE (Dako, USA), CD8 FITC/CD4 PE/CD3 PE-Cy5 (Dako, USA), CD3 FITC/CD(16 + 56) PE, (Beckman Coulter, USA) or CD45 PerCp/CD3 FITC/CD8 PE/CD4 APC (Becton Dickinson, USA) and CD 45 PerCp/CD19 APC/CD3 FITC/CD(16 + 56) PE (Becton Dickinson, USA). After surface staining, erythrocytes were lysed using 1 ml of BD FACSlysing Solution (Becton Dickinson, USA), diluted according to manufacturer's instructions. Remaining cells were washed twice and resuspended in CELL WASH buffer (Becton Dickinson, USA) for further analysis for a FACS Canto II flow cytometer using FACSDiva v7.0 software (Becton Dickinson).

2.3. Droplet digital PCR

Droplet digital PCR was performed using the QX200 Droplet Digital PCR system (Bio-Rad, USA). The 20 µL ddPCR reaction mixture contained ddPCR master mix, 9 µL of tested genomic DNA (6 ng/µL, approximately 2000 copies per reaction), and a 20 × 1 µL primers/probes mix (TUPLE1/RPP30/ZNF74/D22S936, see Table 1) with concentrations as listed in Table 2. The reaction mixtures, along with 70 µL of droplet generation oil, were loaded into a disposable plastic cartridge and placed in the droplet generator. After processing, droplets generated from each sample were transferred to a 96-well PCR plate (Eppendorf, Hamburg, Germany). Amplification was carried out in a T100 Thermal Cycler (Bio-Rad, USA) under the following conditions: DNA polymerase activation at 95 °C for 10 min, followed by 45 cycles of PCR amplification (94 °C for 30 s and 57 °C for 60 s), and a final step of 98 °C for 10 min with a ramp rate of 2 °C/s for all steps. After PCR, the plate was loaded onto the droplet reader, and the acquired data were analyzed using QuantaSoft Analysis Pro software (Bio-Rad, USA).

Table 1
Primers and probes for ddPCR.

Locus	Location	Primer	5'-sequence-3'
TUPLE1	22q11.21	TUPLE1-F	GGCAAGTGCAATATTCATGTGGT
		TUPLE1-R	TCCTACACGCCTGACAAAGCT
		TUPLE1-P	FAM-CCAAAGGTGTTCATTGGAGAGTACTC-BHQ1
ZNF74	22q11.21	ZNF74-F	TGGCCTCCTGCTTCTTCTTC
		ZNF74-R	CAGACACTCCAATTCATGACGAA
		ZNF74-P1	HEX-CCAATCATTGTGCCACTCAGA-BHQ2
D22S936	22q11.21	ZNF74-P2	HEX-CTGCTACCCCAATCAAGCAC-BHQ2
		D22S936-F	TGGCAGCCAGTTTAGTATTCTGC
		D22S936-R	TTGTAATCAAGTCCCGCCACT
		D22S936-P1	HEX-AGACAGGAAGCAGGAAACCACTC-BHQ2
RPP30	10q23.31	D22S936-P2	HEX-CAGGGAGCTGCAGTATTCCTGGT-BHQ2
		RPP30-F	CAGATTGGACCTGCGAGCG
		RPP30-R	GAGCGCTGTCTCCACAAGT
		RPP30-P	FAM-TCTGACCTGAAGGCTCTGCGCG-BHQ1

Locus	10 × primer concentration	10 × probe concentration	Relative fluorescence
TUPLE1	9 μ M	2.5 μ M	FAM low
RPP30	18 μ M	5 μ M	FAM high
ZNF74	9 μ M	2.5 μ M	HEX low
D22S936	18 μ M	5 μ M	HEX high

2.4. Quantitative PCR

Real-time RT PCR was performed in a CFX 96 thermocycler (Bio-Rad, USA) in a total reaction volume of 20 μL, containing a 1 × PCR buffer (65 mM Tris-HCl pH 8.9, 24 mM (NH₄)₂SO₄, 0.05 % Tween 20, 2.5 mM MgCl₂), 0.3 μM primers and 0.1 μM probe for each loci (Table 1), 2 units of Taq-polymerase (Biosan, Russia) and 3–15 ng of genomic DNA as a template. The amplification was performed using the following program: denaturation at 95 °C for 3 min and 50 cycles with denaturation at 95 °C for 10 s, followed by annealing and elongation at 60 °C for 40 s with the registration of fluorescent signals in FAM and HEX channels. Each experiment was conducted in three independent replicates, each run including a no-template control.

2.5. Data analysis

The results of ddPCR were processed using the described procedure. Concentrations of each locus (TUPLE1, ZNF74, D22S936) and the reference gene (RPP30) were determined with QuantaSoft Analysis Pro software (Bio-Rad, USA), followed by the calculation of mean concentration values $\bar{C}_{TUPLE1}$, $\bar{C}_{ZNF74}$, $\bar{C}_{D22S936}$, $\bar{C}_{RPP30}$. For each locus the ratio to the reference gene was determined: $R_{locus}^{RPP30} = \bar{C}_{locus} / \bar{C}_{RPP30}$, where $\bar{C}_{locus}$ is the mean concentration of the target loci, $\bar{C}_{RPP30}$ – mean concentration of RPP30 gene. Deviation of the ratio R_{locus}^{RPP30} from 1 more than 2σ of ratio variations for normal samples (18 %, σ = 9 % for TUPLE1, 24 %, σ = 12 % for ZNF74, 22 %, σ = 119 % for D22S936) served as the mark of the respective locus deletion ($R_{locus}^{RPP30} < 1$).

The results of flow cytometry, ddPCR, and qPCR were analyzed using GraphPad Prism 8.0 software. Statistical analysis was conducted using the Mann–Whitney U test, and findings were considered statistically significant if the p-value was less than 0.05.

3. Results

3.1. Initial screening for PID symptoms and phenotypic characteristics of samples

Patients with suspected primary immunodeficiency were enrolled in the study between 2013 and 2020 from the Allergology and Immunodeficiency Department of G. Speransky Children’s Hospital No. 9 in Moscow. The hospital’s diagnostic laboratory analyzed the immune status and TREC/KREC numbers in collected blood samples. The diagnoses were confirmed using targeted and whole exome sequencing. The nosologies of the primary immunodeficiency group were as follows: DiGeorge syndrome (DGS), DiGeorge-like syndrome, humoral immunity defects including common variable immunodeficiency (CVID) and hypogammaglobulinemia with confirmed and unconfirmed mutations, and X-linked agammaglobulinemia; combined immunodeficiency with associative and syndromic signs including Nijmegen breakage syndrome, Louis-Bar syndrome, and hyper IgE syndrome; phagocytosis defects: chronic granulomatous disease (Table 3). The criteria for enrollment in the PID group were ages ranging from 0 to 18 years and blood sampling conducted before immunoglobulin transfusion. The criterion for non-inclusion was acute inflammatory diseases.

All patients with DiGeorge syndrome had a 22q11.2 deletion confirmed by molecular genetic methods, including *in situ* FISH, and MLPA. The diagnosis was made according to the criteria of the European Society of Immunodeficiency (ESID) for DiGeorge syndrome.

3.2. Optimization of multiplex ddPCR assay for 22q11.21 deletion screening

The presence of 22q11.21 deletions was tested using droplet digital PCR. Loci from the potentially deleted region (TUPLE1, ZNF74, D22S936) were selected based on the work of Hwang et al. [28] to delineate the borders of the most common deletions: approximately 3 Mbp LCR22A-D and other nested deletions, LCR22A-B, LCR22A-C, which are found in approximately 70–80 % and 10–15 % of patients with 22q11.21 deletion syndrome, respectively (see Fig. 1). RPP30 served as a reference single-copy gene, for which no germinal rearrangements are known [32–35].

In this study, we combined three loci, mapping 22q11.2 deletions (TUPLE1, ZNF74, D22S936), with a reference gene (RPP30) in a single tetraplex assay. This design enabled the quantification of concentrations of all three marking loci in a single tube for one sample, while simultaneously determining the genomic DNA amount via counting the reference gene copies. Multiplexing was achieved using a ratio-based approach, wherein different concentrations of probes for various targets were employed. Consequently, targets were identified based on the fluorescence amplitude of corresponding clusters. Four independent targets (TUPLE1, ZNF74, D22S936, RPP30) were amplified simultaneously in one reaction, with two probes for each target having similar fluorophores (e.g., FAM or HEX). The probes with the same fluorophore

Nosology	Males	Average age, years	Females	Average age, years
DiGeorge syndrome	45	4.79±0.89	37	4.78±1.01
DiGeorge-like syndrome	13	4.51±1.25	2	5.89±2.51
Humoral immunity defects	12	11.19±1.60	1	16.02
Combined immunodeficiency	2	5.63±2.15	4	7.33±1.85
Undefined primary immunodeficiency*	19	11.51±5.56	17	6.23±3.81
Phagocytosis defects	1	1.27	0	–
All	91	6.44±0.75	61	5.75±0.88

* — patients with primary immunodeficiency unmatching all other listed categories or without defined genetical cause.

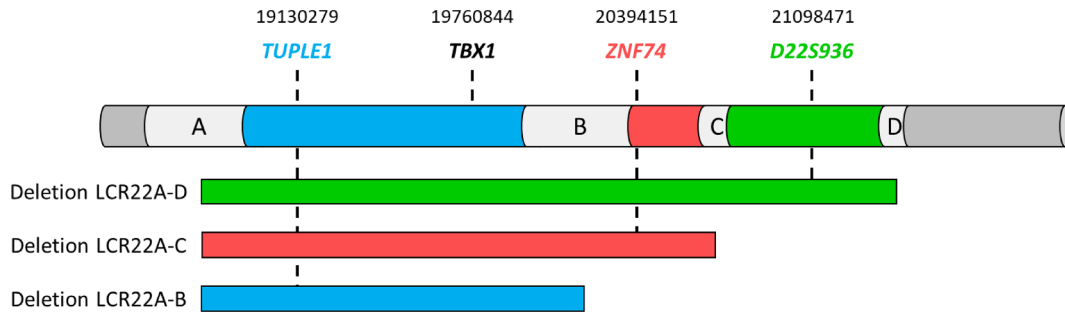


Fig. 1. Schematic presentation of various deletions and loci used for the multiplex ddPCR assay. A, B, C, D represent low copy repeats, while *TBX1*, *TUPLE1*, *ZNF74*, and *D22S936* denote specific loci. The numbers indicate the genomic coordinates of these loci.

were added to the reaction mix in a 2:1 ratio, resulting in different amplitudes of endpoint fluorescence for each target. Depending on the template's amount, up to 16 clusters of droplets could be formed, each containing from 0 to all four targets. Comparison of concentrations between mapping loci and the reference gene allowed us to determine the presence and borders of 22q11.21 deletions in the analyzed DNA specimen. The overall analysis process is presented at Fig. 2.

Initially, primers and probes for the amplification of mapping loci were sourced from the work of Hwang et al [28]. The reference gene *RPP30* has been previously utilized in several studies for the quantification of human genomic DNA [34–36]. All primers were individually tested in monoplex quantitative PCR, and effective amplification with the respective probe was confirmed. However, when all four sets of primers and probes were multiplexed in one ddPCR reaction, it resulted in ineffective clusterization (see Fig. 3). Clusters formed by *ZNF74*- and *D22S936*-positive droplets were indistinguishable, making DNA quantification impossible.

One possible explanation for the clusterization issues could be the abnormal fluorescence of the probes. It was observed that the baseline fluorescence of probes for *ZNF74* and *D22S936* (*ZNF74*-P1, *D22S936*-P1) loci in digital PCR was twice as high as the typical fluorescence

amplitude for the given probe concentration. The baseline fluorescence in negative clusters for both HEX targets was approximately 7000 RFU instead of the expected range of 2000–2500 RFU. This discrepancy could arise from low-quality synthesis of oligonucleotides, wherein unattached fluorophore molecules remain in the oligonucleotide stock solution after purification, or from a high percentage of probes lacking a quencher. Another potential explanation could be the spatial structure of the probe, where the fluorophore and quencher are distanced from each other, hindering quenching.

Initially, we attempted to adjust the concentration of a HEX-labelled probe for *ZNF74* to achieve readable results with clearly distinguishable clusters. In the first attempt, the probe was added in a ratio of 1:2 with the *D22S936* probe, resulting in concentrations of 125:250 nM, respectively. To achieve a difference of approximately 2000 RFU between HEX clusters, we calculated a concentration of the *ZNF74* probe as 190 nM based on the initial and final fluorescence of HEX-labelled probes. This calculation assumed a linear dependence between a probe's concentration and its fluorescence when final probe concentrations are in the range of 50–300 nM. A concentration of the *ZNF74* probe two times lower than the standard concentration (75 nM) was also tested. However, the results obtained did not show improvement

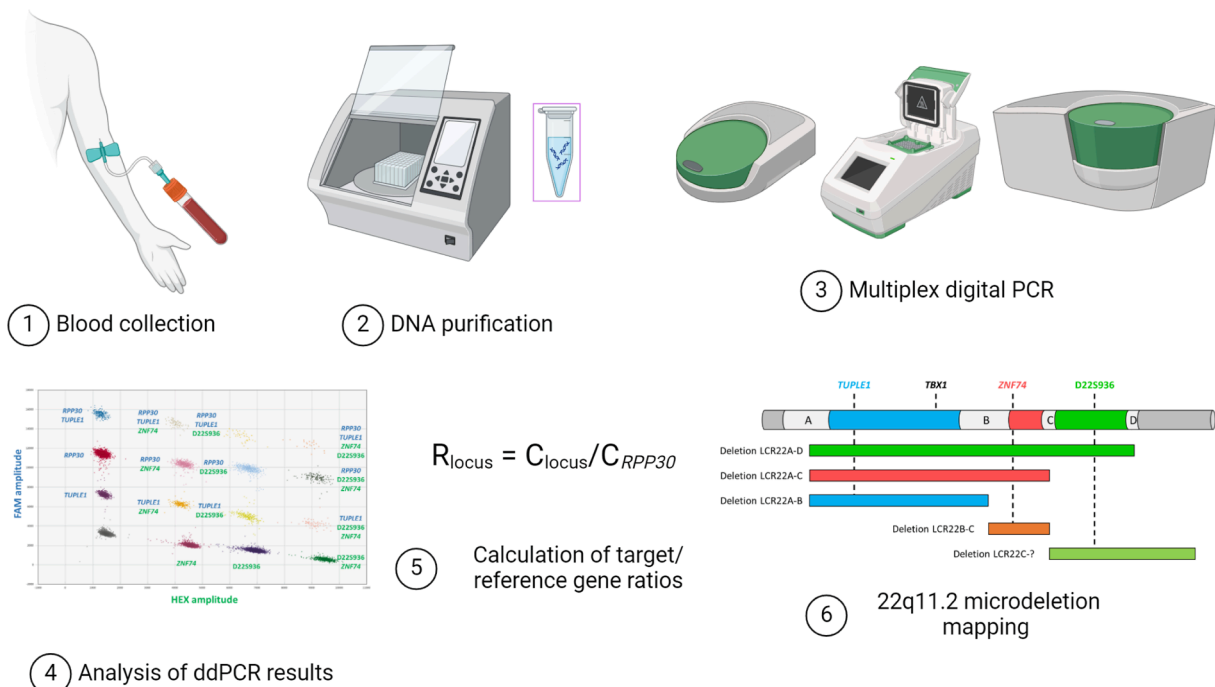


Fig. 2. Flowchart of 22q11.2 microdeletions analysis using multiplex ddPCR. After blood collection, DNA is purified from nucleated blood cells and serves as a template for multiplex ddPCR. For each locus the ratio to the reference gene was calculated. When ratios are close to 0.5, the sample is considered as 22q11.2 microdeletion positive and the microdeletion is mapped using targeted loci (biorender.com).

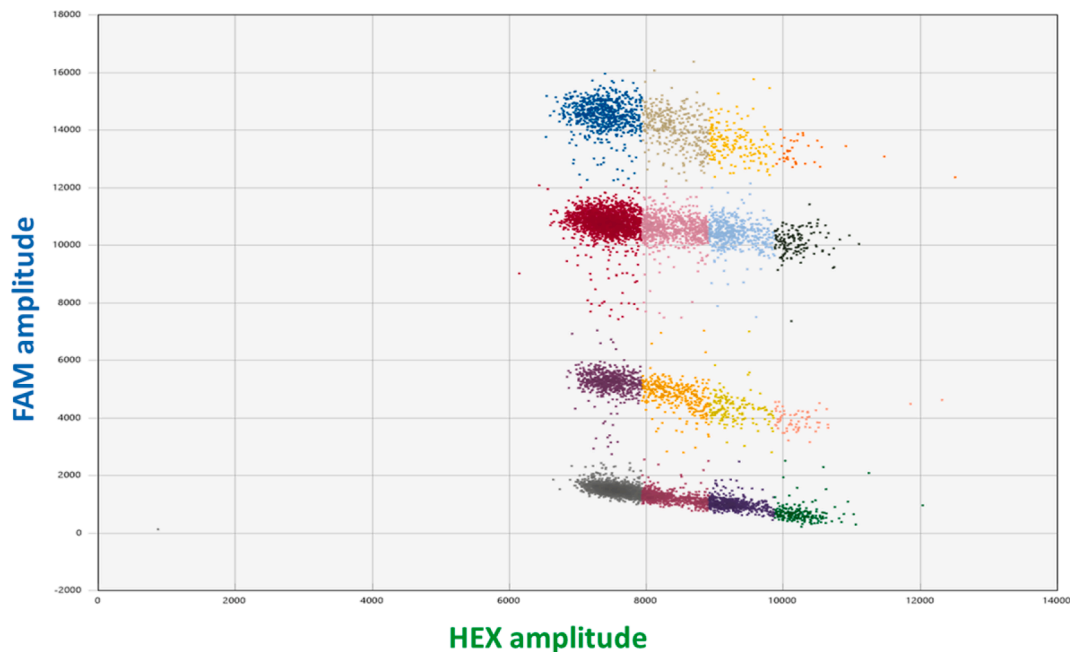


Fig. 3. Clusterization of the initial multiplex digital PCR. The results of ddPCR are presented as 2D-graphs, where FAM fluorescence amplitude is plotted on the X-axis, and HEX fluorescence amplitude is plotted on the Y-axis. Colors are used to mark different putative clusters.

compared to the standard concentration of 125:250 nM, as the HEX clusters remained too close to each other without a significant difference.

Redesigning the probes became an alternative approach to address the problem, and new probes (ZNF74-P2, D22S936-P2) were selected on other DNA strands from the initial ones. The new probes were assessed at the standard ratio of 1:2 (125:250 nM) and provided proper clusterization, allowing quantification of the respective targets (see Fig. 4). Thus, the second set of HEX-labelled probes was used in the following experiments.

Finally, we titrated a template — human genomic DNA — in the

range of $32-2 \times 10^4$ copies per reaction to evaluate the sensitivity of the developed multiplex ddPCR assay. DNA samples with and without confirmed 22q11.2 microdeletion served as templates for the titration experiment. The results of the titration are presented in Fig. 5.

The graphs in Fig. 5 demonstrate that the ddPCR assay allows for the detection of 22q11.2 microdeletions when approximately 32 copies of the template are added to the reaction mix. The amount of DNA purified from two dried blood spots falls within the range of 2.16–24 ng/ μ L or 7,128–79,200 copies/ μ L [37]. Therefore, tetraplex digital PCR could be utilized for neonatal screening of 22q11.2 microdeletions using dried blood spots from infants.

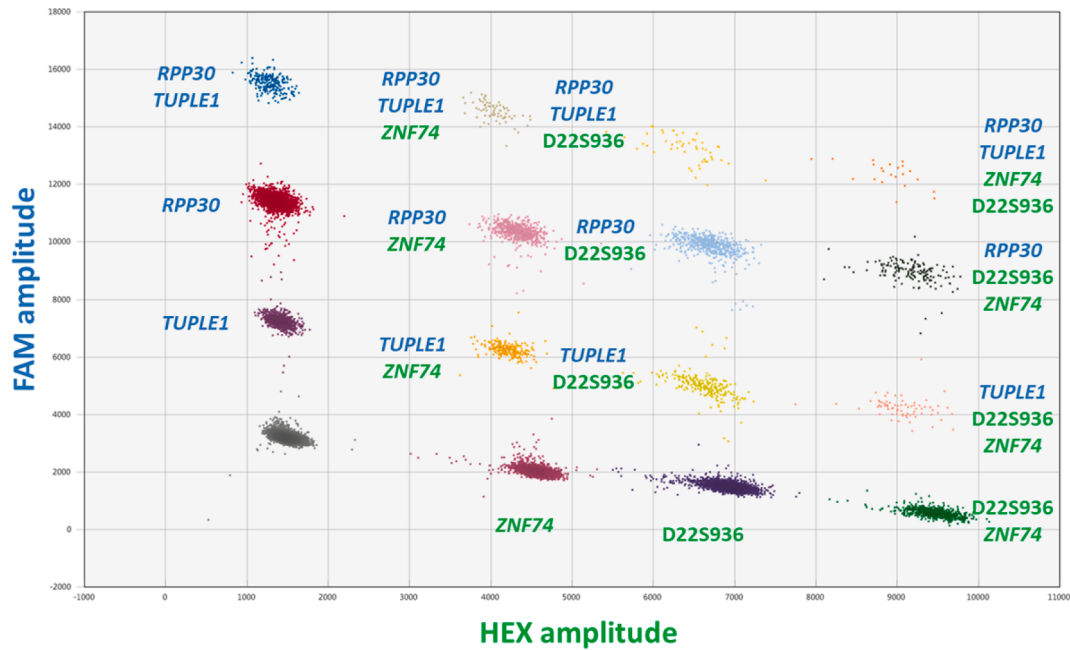


Fig. 4. Clusterization of final multiplex digital PCR. The results of ddPCR are presented as 2D-graphs, with FAM fluorescence amplitude is plotted on the X-axis and HEX fluorescence amplitude is plotted on the Y-axis. Each cluster is designated and marked by colors indicating the targets. Target colors correspond to fluorophore types: blue for FAM and green for HEX.

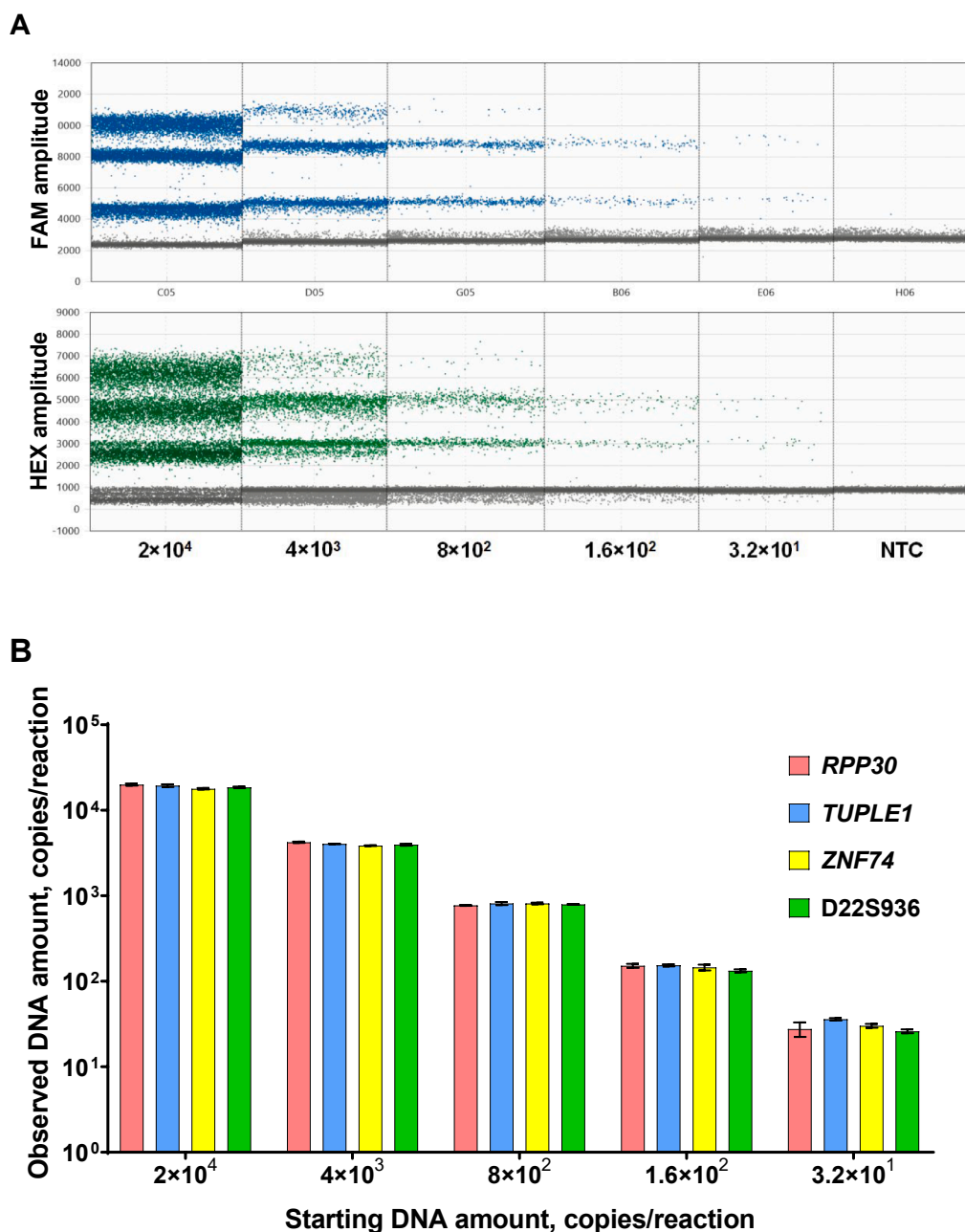


Fig. 5. Template titration in multiplex ddPCR. The results of ddPCR are depicted as 2D-graphs of clusterization, with FAM and fluorescence amplitudes are plotted on the X-axis and DNA concentration in samples is plotted on the Y-axis. Each cluster is designated and marked by colors indicating the targets. Target colors correspond to fluorophore types: blue for FAM and green for HEX.

3.3. Analysis of clinical samples using multiplex ddPCR

A total of 153 samples taken from patients with DGS-like symptoms were analyzed using the optimized multiplex ddPCR assay. The obtained data were utilized to calculate the ratios between the mean concentrations of each marking locus and the reference gene *RPP30* (refer to the Materials and Methods section). A difference from the mean ratio by more than two standard deviations (SD) (see Table 4) was established as the cutoff for a positive decision regarding the deletion of a particular marking locus. Graphical results of the validation are presented in Fig. 6.

Almost all samples were successfully divided into four groups: wild-type with no microdeletion, LCR22A-B, LCR22A-C, and LCR22A-D deletions. As expected, the ratio of mapping loci to *RPP30* was close to 1 or 0.5, depending on the presence of deletions. The longer microdeletion

means the more loci were affected, resulting in a low ratio with *RPP30*. The most frequent microdeletion was LCR22A-D, while LCR22A-B and LCR22A-C deletions were much less common. However, in three samples, we found uncommon alterations. In one sample, only the *ZNF74* ratio was 0.665, while the ratios for *TUPLE1* and *D22S936* were 0.838 and 0.752, respectively. Plausibly, these results indicate the presence of an LCR22B-C deletion. In the other two samples, we observed a lower ratio only for *D22S936*, which hints at distal deletions with a 5'-end at the LCR22D repeat. Since our assay covers only proximal deletions, the 3'-end of these alterations remained unknown.

After analyzing clinical samples using multiplex ddPCR, we tested the samples by quantitative PCR (qPCR) with primers and probes optimized for ddPCR. Previously, several groups published qPCR-based assays for the 22q11.2 microdeletion [27,38–40]. Here, we assumed that

Table 4

Sample type	Number of samples	Mean (CI 95 %)		
		TUPLE1	ZNF74	D22S936
Wild-type	67	0.964 (0.943–0.985)	0.946 (0.921–0.972)	0.954 (0.930–0.977)
Cut-off (2 × SD)		0.172	0.208	0.195
LCR22A-B	6	0.509 (0.459–0.559)	1.043 (0.897–1.19)	1.041 (0.937–1.14)
LCR22A-C	2	0.496	0.535	0.963
LCR22A-D	74	0.476 (0.465–0.487)	0.448 (0.429–0.466)	0.462 (0.447–0.476)
LCR22B-C	1	0.838	0.665	0.752
LCR22C-?	2	0.850	0.884	0.592
Overall	153			

primers and probes optimized for ddPCR would be effective in qPCR and used these oligonucleotides for the qPCR assessment of clinical samples. Each sample was analyzed in triplicate, and each run included a calibration curve and a non-template control. The calibration curves were prepared using human genomic DNA titrated in the range of $32\text{--}2 \times 10^4$ copies per reaction. To avoid optimization and assessment of multiplex qPCR, each locus was quantified in separate monoplex qPCR reactions. The results of the qPCR-based test are provided in Fig. 7.

For TUPLE1 and D22S936, wild-type samples and samples with the LCR22A–D microdeletion were clearly distinguishable from each other.

Unlike ddPCR, the efficacy of qPCR with primers and probes for ZNF74 was around 80 % (data not shown), which led to the absence of a difference between the two types of samples. It is plausible that other reaction conditions could provide better results for ZNF74 and the other two loci, but optimization is a separate task that was beyond the scope of the present study. qPCR can be a method of choice when the cost per analysis is crucial. However, even well-optimized and multiplexed qPCR has inherent limitations: the need for external standards, a higher limit of quantification, and lower accuracy compared to ddPCR. The latter is advantageous for the analysis of low-abundance DNA samples, such as DNA from dried spots.

3.4. Correlation analysis between the deletion presence and blood parameters

At the final stage of the present study, we investigated a potential correlation between the presence of a microdeletion and several blood characteristics, including cell counts and the number of excision circles from T-cell (TREC) and B-cell (KREC) receptors. Blood parameters were assessed by flow cytometry, while excision circles were counted by qPCR, followed by a comparison between various deletions and the wild-type. We took into account only LCR22A–D deletion because other deletions were rare and could not be reliably analyzed.

The results for TREC and KREC numbers, leukocytes, and lymphocytes are shown in Fig. 8.

Based on the obtained results, only TREC numbers correlated with

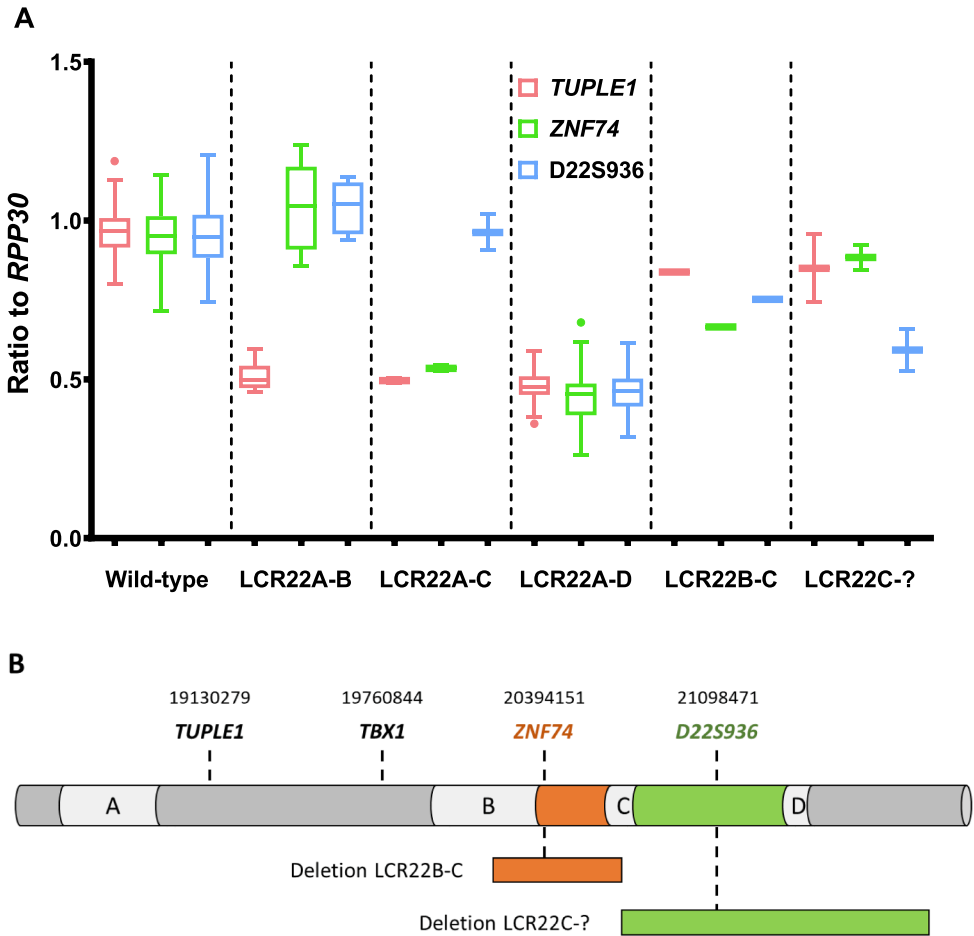


Fig. 6. ddPCR analysis of clinical samples. A. Ratios of mapping loci with RPP30. The Y-axis represents the ratios of mapping loci to the reference gene RPP30, while the X-axis denotes various 22q11.2 deletions or their absence. Mapping loci are indicated by color, as shown in the legend. B. Schematic presentation of uncommon deletions and loci used for the multiplex ddPCR assay. A, B, C, D represent low copy repeats, while TBX1, TUPLE1, ZNF74, and D22S936 denote specific loci. The numbers indicate the genomic coordinates of these loci.

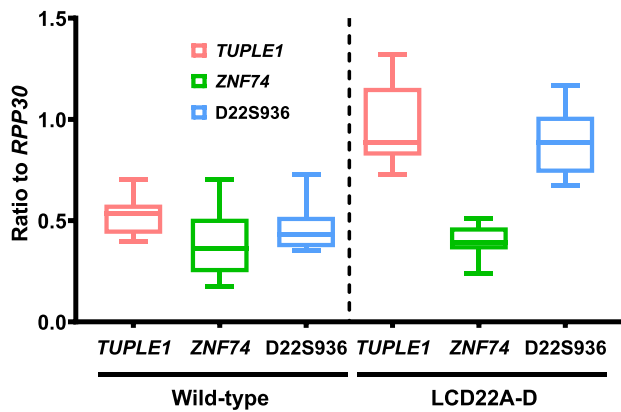


Fig. 7. QPCR analysis of clinical samples. The Y-axis represents the ratios of mapping loci to the reference gene RPP30, while the X-axis denotes LCD22A–D microdeletion or its absence. Mapping loci are indicated by color, as shown in the legend.

the presence of the LCD22A–D deletion with a p-value <0.005. Neither leukocyte nor lymphocyte counts, nor KREC numbers, were associated with the microdeletion, suggesting that the main affected immune cell population is T-cells maturing in the thymus.

Furthermore, we assessed lymphocyte subpopulations in relation to the presence of the microdeletion, quantifying cells and calculating their percentages (Fig. 9).

In the presence of LCD22A–D microdeletion, cell counts of CD3+ lymphocytes (all T-cells), CD4+ cells (T-helpers), and CD8+ cells (T-killers) remained unchanged compared to wild-type samples. However, counts of CD19+ cells (B-lymphocytes) and natural killer cells significantly increased with p-values of 0.038 and 0.0009, respectively. Conversely, when comparing lymphocyte percentages, there was a notable shift. While fractions of all T-cells, T-killers, and T-helpers decreased, B-cells and, especially, natural killer cells became more abundant. It should be noted that the confidence level of these changes was highest for T-cells, T-helpers, and natural killer cells, with p-values lower than 0.005.

However, the difference between samples from the DiGeorgi-like syndrome group and with LCD22A–D microdeletion was much less striking (Supplementary Fig. 1 A–C). Counts of B-cells were similar in these two groups, as well as the percentage of T-killers, B-cells and natural killers. NK counts was higher (p-value = 0.0179), while the

percentage of T-helpers, T-cells was lower in samples with LCD22A–D microdeletion (p-value = 0.0223, 0.0383, respectively).

4. Discussion

While fluorescence *in situ* hybridization (FISH) remains a common method for diagnosing DiGeorge syndrome (DGS), several alternative approaches have been explored, including multiplex ligation-dependent probe amplification (MLPA), quantitative polymerase chain reaction (qPCR), and digital PCR [28,41,42]. Digital PCR offers the advantage of precise and direct counting of DNA molecules, but its straightforward application often results in a large number of individual reactions corresponding to the number of loci under study. This not only increases the cost of analysis but also makes the process cumbersome. An effective strategy for overcoming this issue is the multiplexing of several digital PCR reactions in a single tube.

Two main approaches can be used for the multiplexing of digital PCR [43]. The ratio-based approach involves using two TaqMan probes for a single target with different fluorophores, resulting in a single cluster with simultaneous fluorescence at both channels [29,44]. The amplitude-based approach, on the other hand, entails using different concentrations of TaqMan probes with a similar fluorophore for different targets, leading to clusters with distinct fluorescence magnitudes [45,46]. When four targets are multiplexed in a single tube, the amplitude-based approach typically produces a 2D-graph with easily recognizable 16 clusters arranged rectangularly. Based on our prior experience with multiplex digital PCR, the amplitude-based multiplexing approach is relatively straightforward to design and optimize, and it has been successfully applied for detecting rearrangements in the *BRCA1* gene [36].

In our previous work with *BRCA1* deletions [36], we encountered incomplete clusterization, which was addressed by redesigning probes for two targets. It is noteworthy that the initial set of probes not only resulted in merged clusters but also exhibited baseline fluorescence signals higher than usual. The exact reason for this phenomenon remains unclear, but two speculations can be made. Firstly, it could be due to sequence-specific interactions between the probes and other oligonucleotides in the reaction mix. Secondly, it could be attributed to sub-optimal synthesis of the probes themselves. Further investigations are warranted to develop a robust protocol for the design and validation of ddPCR primers and probes.

Our analysis of clinical samples demonstrated the high prevalence of the typical LCD22A–D microdeletion which is agreement with other

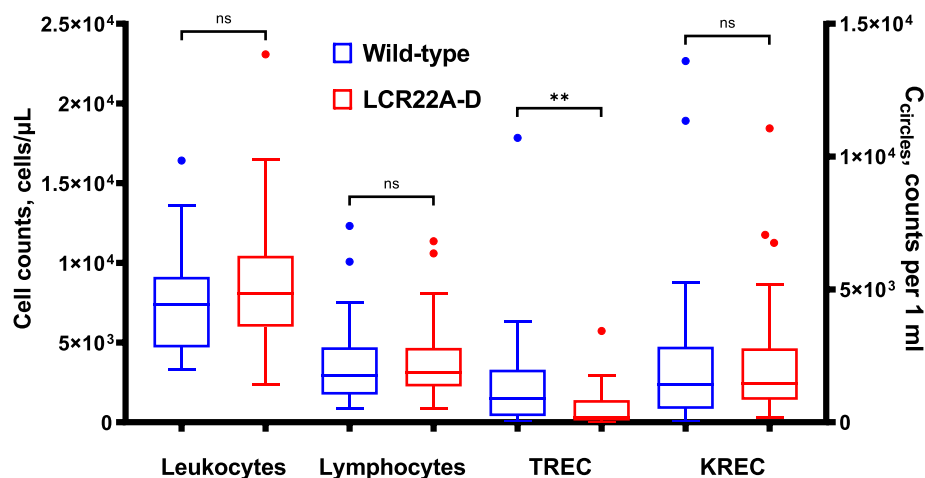


Fig. 8. Blood cell counts and excision circles in wild-type and microdeletion samples. The left Y-axis represents the cell counts in blood samples, the right Y-axis represents TREC and KREC numbers, and the X-axis denotes the presence or absence of LCD22A–D deletions. Asterisk symbols indicate the confidence level, **P<0.005, ns — not significant.

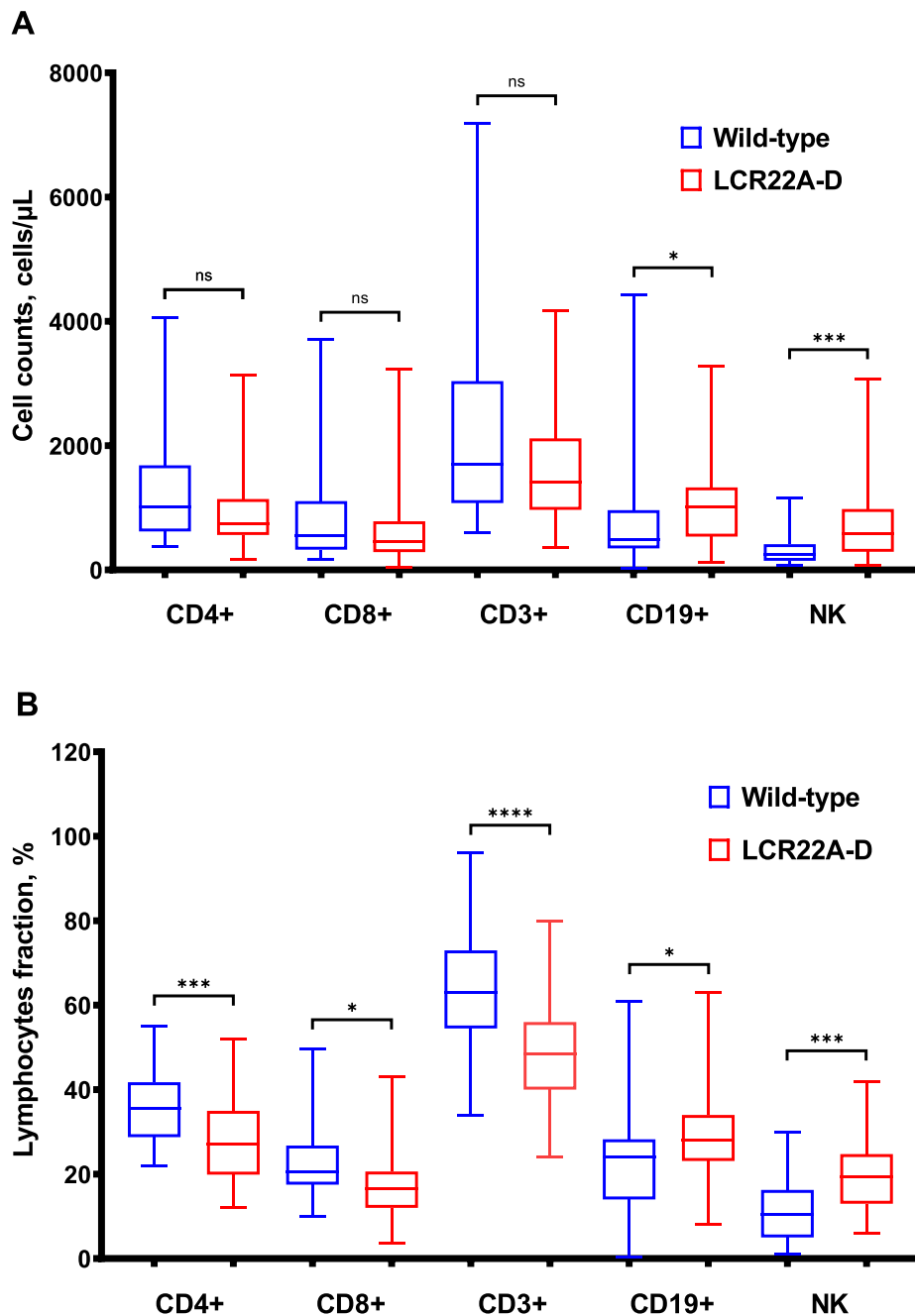


Fig. 9. Lymphocyte subpopulations in wild-type and microdeletion samples. A — cell counts, B — fraction percent. The Y-axis represents the cell counts or fraction percent in blood samples, and the X-axis denotes the lymphocyte subpopulations. Asterisk symbols indicate the confidence level, * $P < 0.05$, *** $P < 0.0005$, **** $P < 0.00005$, ns — not significant.

works. Microdeletions located between other repeats were much less common [15,47]. However, two distal deletions were found starting from the LCR22C. Previous studies reported these microdeletions as relatively rare events with clinical manifestation different from classical proximal microdeletions [17]. Here, we were not able to define borders of distal alterations because our digital PCR assay encompasses only the LCR22A-D region. In that sense, other loci located between LCR22D-H would be necessary for more representative screening analysis.

The results of cell counting were different from what was previously reported by other groups. Counts of B-cells and natural killers were stated to remain the same despite the presence of microdeletions [48,49]. However, their functioning was also impaired due to the T-lymphocyte disorder affecting other immune cells. Here, we found

statistically higher levels of B-cells and natural killers in patients with microdeletions comparing to patients with PID and wild-type genotype. This difference can be a consequence of the low level or absence of the respective cell types in patients with several specific PIDs, including CVID, X-linked agammaglobulinemia, and Nijmegen breakage syndrome. If these patients were excluded from the analysis, and only subjects with DiGeorge-like syndrome were considered, this latter wild-type genotype group was less different from the group with LCR22A-D deletions. Only the difference in CD3+ and CD4+ cell percentages was noted in that comparison (Supplementary Figure 1).

As limitations of our approach, several issues need to be mentioned. Firstly, droplet digital PCR on the QX platform is more prone to contamination than qPCR, and its usage requires special precautions,

such as time and space management of processed samples. It should be noted, though, that the developed ddPCR assay works with a relatively high amount of DNA isolated from blood or blood spots, which decreases the risk of false results caused by contamination from previous runs. Secondly, our ddPCR cannot map distal microdeletions located outside of the region between LCR22A-LCR22D. These microdeletions will remain undetected. However, these rearrangements lead to clinical symptoms different from the classical picture and probably can be designated as a different clinical entity. Thirdly, our frequency results concerning the frequency of 22q11.21 microdeletions cannot be taken into account for the calculation of their presence in the broad Russian population because we only analyzed patients with already manifesting immune disorders.

5. Conclusion

To sum up, we present here a simple and economical method for DiGeorge screening based on droplet digital PCR. The assay involves multiplex amplification of four loci located in the most frequently deleted region between LCR22A and LCR22D repeats. The designed method was optimized and validated on samples taken from children showing signs of immunodeficiency, with the LCR22A-D microdeletion being the most common among other alterations. The described assay is suitable for clinical practice and represents a vital alternative to more expensive NGS and less accurate phenotype examinations.

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Author contributions

I.P.O.: Methodology, Formal analysis, Writing – Original Draft, Writing – Review & Editing, **M.A.G.:** Resources, Writing – Review & Editing, **N.V.D.:** Resources, Writing – Review & Editing, **N.V.Z.:** Resources, **E.F.K.:** Resources, **L.A.:** Resources, **D.A.K.:** Funding acquisition, **M.L.F.:** Conceptualization, Writing – Review & Editing, Project administration. All authors read and approved the final version of the manuscript. I.P.O. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

CRediT authorship contribution statement

Igor Petrovich Ocorbin: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Maria Alexandrovna Gordukova:** Writing – review & editing, Resources. **Nataliia Vladimirovna Davydova:** Writing – review & editing, Resources. **Natalia Valentinovna Zinovieva:** Resources. **Elena Fedorovna Kovzel:** Resources. **Lucia Andries:** Resources. **Dmitry Anatolyevich Kudlay:** Funding acquisition. **Maxim Leonidovich Filipenko:** Writing – review & editing, Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary material to this article can be found online at <http://dx.doi.org/10.1016/j.cca.2024.119903>.

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