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PRECLINICAL STUDY

Multiplex ddPCR assay for screening copy number variations in *BRCA1* gene

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Abstract

Purpose Germinal and somatic rearrangements in *BRCA1* gene play a significant role in carcinogenesis of breast and ovarian cancer. The present study is dedicated to the development of multiplex droplet digital PCR (ddPCR) assay for detecting large deletions and duplications in the *BRCA1* gene.

Methods In-house tetraplex ddPCR assay for *BRCA1* gene analysis was used for testing of DNA samples with *BRCA1* status.

Results DNA specimens were purified from 24 individuals. The presence of *BRCA1* rearrangements in samples was confirmed by a commercial MLPA-based kit. An amplitude-based multiplex ddPCR assay was developed: 8 multiplexes, each containing primers and probes to amplify 3 *BRCA1* exons and 1 reference gene (*ALB* or *RPP30*). A novel assay demonstrated 100% concordance with the commercial MLPA-based kit, identifying 9 specimens with different deletions in *BRCA1*, 1 with duplication, and 14 with the wild-type *BRCA1*.

Conclusions We have designed a simple, precise, and cost-effective assay for *BRCA1* rearrangement testing, based on ddPCR. The developed assay is the first multiplex ddPCR-based test that provides results in accordance with MLPA and can be used for routine clinical screening.

Keywords *BRCA1* · Digital PCR · Gene rearrangements · CNV · Multiplex amplification

Introduction

BRCA1 gene is one of the two genes frequently mutated in breast and ovarian cancer [1]. Mutations that violate functions of encoded protein have clinical significance and commonly classified as deleterious. They can be germinal and increase the risk of cancer in a patient and carrier relatives as well as a somatic mutation that transforms this mutant cell into a tumor cell. The presence of a deleterious germinal or somatic mutation in a tumor could be used for the selection of targeted therapy [2]. Tumor cells carrying the *BRCA1* mutation possess a reduced capability to repair double-stranded breaks leading to the high efficiency of drugs breaking other mechanisms of DNA repair (e.g., olaparib). Consequently, the detection of clinically significant mutations in the *BRCA1* gene may be useful in two ways: to identify patients with a high risk of cancer and to choose an effective way for tumor treatment.

The functionality of *BRCA1* protein can be disrupted by point mutations as well as large rearrangements in its gene. Short (less than the length of an exon) BRCA mutations are identified in 15% [3, 4] germline and 1.5–2% [5–7] somatic condition in ovarian or breast tumors. Germline large rearrangements (LRs), or copy number variations (CNVs), occur in about 1–2% of the ovarian cancer cases [3, 4], making up a significant part of all cases with *BRCA* mutations (~ 10%). The frequency of somatic CNVs in the *BRCA1* gene was described in only one work, with the occurrence of 27% among patients with ovarian cancer [8].

Before the recent advent of digital droplet PCR (ddPCR), multiplex ligation-dependent probe amplification (MLPA) was thought to be the most reliable method for detecting CNVs in medically relevant genes such as *BRCA1*. To date, with ddPCR, we have an opportunity to perform the absolute

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quantification of the number of allele copies and to compare them easily [9]. For past several years, digital PCR has been used in a number of applications, including detection of GMO [10], nucleic acids of pathogens [11, 12], somatic mutations [13, 14], and tracing hereditary disorders, which are caused by genomic rearrangements [15, 16]. Preobrazhenskaya and coauthors have already described the usage of ddPCR for the detection of the *BRCA1* CNVs [17]. However, the approach described includes a large number of reactions which are rather time consuming and do not fit clinically grade tests.

We have developed a mid-throughput ddPCR method to identify CNVs in the *BRCA1* gene using tetraplex reactions that cover all coding and non-coding exons and two reference gene regions (*RPP30* and *ALB*) and tested its performance characteristics using real clinical DNA samples.

Materials and methods

Patients and samples

Samples were collected from patients with serious ovarian cancer receiving diagnostic support under the program "Improving the system of molecular genetic diagnosis of cancer in the Russian Federation" (http://www.cancergenome.ru). All patients signed an informed written consent to participate and to have their biological specimens analyzed. All procedures performed in the studies involving human participants were conducted in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

DNA was extracted from blood samples using QIAamp DNA Blood Maxi Kit (Qiagen, Germany) in accordance with the manufacturer's protocol. As a reference method for testing of *BRCA1* rearrangements, we used multiplex ligase-dependent probe amplification (MLPA). MLPA analysis was performed using the SALSA P002 and P087 BRCA1 MLPA Kits according to the manufacturer's instructions (MRC-Holland, the Netherlands).

Real-time PCR

Real-time PCR assays were performed in 20 µL containing 65 mM Tris–HCl, pH 8.9, 24 mM $(NH_4)_2SO_4$, 0.05% Tween-20, 3 mM $MgSO_4$, 0.2 mM dNTP, 300 nM primers, 100 nM hydrolysing probe (Table 1), 30 ng of human genomic DNA from blood, and 1 U of Taq-polymerase (Biosan, Russia). Amplification was carried out in the CFX96 Real-Time PCR Detection System (Bio-Rad, USA) according to the following program: 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, and 60 °C for 40 s with a collection of fluorescent signals at the respective channel.

Droplet digital PCR

Droplet digital PCR was performed with the QX100 Droplet Digital PCR system (Bio-Rad, USA). The ddPCR reaction mixture consisted of 10 µL of 2 × ddPCR master mix (Bio-Rad, USA), 400 genome-equivalents of tested DNA, if another amount is not specified, and 20 × primers/probes mix (Table 1) in a final volume of 21 µL. The entire reaction mixture was loaded into a disposable plastic cartridge (Bio-Rad, USA) together with 70 µL of droplet generation oil (Bio-Rad, USA) and placed into the droplet generator (Bio-Rad, USA). After processing, the droplets generated from each sample were transferred to a 96-well PCR plate (Eppendorf, Germany). PCR amplification was carried out on a T100 Touch thermal cycler (Bio-Rad, USA) using a thermal profile beginning at 95 °C for 10 min, followed by 45 cycles of 94 °C for 10 s, and 57 °C for 60 s, and ending of 98 °C for 10 min at a ramp rate of 2 °C/s. After PCR, the plate was loaded on the droplet reader (Bio-Rad, USA); acquired data were analyzed with QuantaSoft Analysis Pro software (Bio-Rad, USA).

Data analysis

Results of ddPCR were processed in accordance with the described procedure. Concentrations of each exon and two reference genes (*ALB*, *RPP30*) were determined with QuantaSoft Analysis Pro software (Bio-Rad, USA) followed by calculation of mean concentration values $\bar{C}_1, \bar{C}_2, \ldots, \bar{C}_{24}, \bar{C}_{ALB}, \bar{C}_{RPP30}$. For each exon two ratios were counted, separately for two reference genes: $R_n^{ALB} = \bar{C}_n / \bar{C}_{ALB}$ and $R_n^{RPP30} = \bar{C}_n / \bar{C}_{RPP30}$, where $\bar{C}_n$ is the mean concentration of the n exon, $\bar{C}_{ALB}$ is the mean concentration of *ALB* gene, $\bar{C}_{RPP30}$ is the mean concentration of *RPP30* gene. Deviation of both ratios R_n^{ALB} and R_n^{RPP30} from 1 more than 2σ of ratio variations for normal samples (20%, $\sigma = 9.3\%$) served as mark of the respective *BRAC1* exon deletion ($R_n^{ALB} < 1$, $R_n^{RPP30} < 1$) or duplication ($R_n^{ALB} > 1$, $R_n^{RPP30} > 1$).

Results

Oligonucleotide primers and probes design

In the present work, we decided to divide 24 exons of *BRCA1* gene into 8 groups along with one or two reference gene for quantification. The design allowed quantifying concentrations of all 24 *BRCA1* gene exons in 8 reactions

Table 1 Primers and probes used for multiplex ddPCR assay

Target	Primer	5-′sequence-3′
RPP30 gene	RPP-F	GATTTGGACCTGCGAGCG
	RPP-R	GCGGCTGTCTCCACAAGT
	RPP-PF	FAM-TCTGACCTGAAGGCTCTGCGCG-BHQ1
	RPP-PH	HEX-TCTGACCTGAAGGCTCTGCGCG-BHQ2
ALB gene	ALB-F	GACTTGCCAAGACATATGAAACC
	ALB-R	TCCAACAATAAACCTACCACTTTG
	ALB-PF	FAM-TGCTGTGCCGCTGCAGATCC-BHQ1
	ALB-PH	HEX-TGCTGTGCCGCTGCAGATCC-BHQ2
Exon 1	Ex1-F	GTAATTCCCGCGCTTTTCC
	Ex1-R	CCTTGATTTCGTATTCTGAGAGGCT
	Ex1-P	FAM-CTGCTTAGCGGTAGCCCCTTGG-BHQ1
Exon 2	Ex2-F	GGAATCCCAAATTAATACACTCTTG
	Ex2-R	TGTCATTAATGCTATGCAGAAAATC
	Ex2-P	HEX-TGCTGACTTACCAGATGGGACACT-BHQ2
Exon 3	Ex3-F	AACTTACTTGCAAAATATGTGGTCA
	Ex3-R	CCTACCCTGCTAGTCTGGAGTT
	Ex3-P	HEX-ACTTTGTGGAGACAGGTTCCTTGAT-BHQ2
Exon 4	Ex4-F	GATTTTGCATGCTGAAACTTCTCA
	Ex4-R	CCTTTTGGTTATATCATTCTTACATA
	Ex4-P	FAM-CCAGAAGAAAGGGCCTTCACAG-BHQ1
Exon 5	Ex5-F	TCATTCTTGGGATATTCAACACTT
	Ex5-R	GCTATTGAAAATCATTTGTGCTTT
	Ex5-P	HEX-ACTCCAAACCTGTGTCAAGCTGA-BHQ2
Exon 6	Ex6-F	GGATTTTCGGGTTCACTCTGT
	Ex6-R	GTTTCTATCATCCAAAGTATGGGCT
	Ex6-P	FAM-CAGAAACCGTGCCAAAAGACTTC
Exon 7	Ex7-F	GACAGACGTCTTTTGAGGTTGT
	Ex7-R	TCTCTAACCTTGGAACTGTGAGA
	Ex7-P	HEX-ATCCGCTGCTTTGTCCTCAGAG-BHQ2
Exon 8	Ex8-F	ACCAGCTTCATAGACAAAGGTTCTC
	Ex8-R	GGATCTGATTCTTCTGAAGATACCGT
	Ex8-P	FAM-TTGACTCACCTGCAATAAGTTGCC
Exon 9	Ex9-F	TTTTGCAGAATCCAAACTGATTTC
	Ex9-R	TGTGGGAGATCAAGAATTGTTACA
	Ex9-P	FAM-CCTGGTTCCTTGAGGGGTGAT
Exon 10	Ex10-F	GGTAACCCTGAGCCAAATGTGT
	Ex10-R	GCGTCCAGAAAGGAGAGCTTAG
	Ex10-P	FAM-CAGGAGTCCTAGCCCTTTCACCC
Exon 11	Ex11-F	GATAGCCCTGAGCAGTCTTCAGA
	Ex11-R	TTGTTATTTAAGGTGAAGCAGCATC
	Ex11-P	FAM-ACGCTTGTTTCACTCTCACACCC
Exon 12	Ex12-F	TGATGGAAGGGTAGCTGTTAGA
	Ex12-R	GCAGGAAATGGCTGAACTAGA
	Ex12-P	HEX-CTGGCTCCCATGCTGTTCTAAC-BHQ2
Exon 13	Ex13-F	GACACCTCAAACTTGTCAGCAGA
	Ex13-R	GCAGTATTAACTTCACAGAAAAGTAGTGA
	Ex13-P	HEX-CCCTATAAGCCAGAATCCAGAAGGC
Exon 14	Ex14-F	AATCGTGTGGCCCAGACT
	Ex14-R	CCCATCTCAAGAGGAGCTCA
	Ex14-P	FAM-TGCTCCTCCACATCAACAACCTTAA-BHQ1

Table 1 (continued)

Target	Primer	5-′sequence-3′
Exon 15	Ex15-F	TCAGGGTCATCAGAGAAGAGG
	Ex15-R	CCCATTCCTTTCAGAGGGAA
	Ex15-P	FAM-CCCAGAGTCAGCTCGTGTTGG-BHQ1
Exon 16	Ex16-F	AGATTAGTTAAAGTGATGTGGTGTTTTC
	Ex16-R	GAGGTAACTCATGATAATGGAATATTTG
	Ex16-P	HEX-CAGATGCTCGTGTACAAGTTTGCC
Exon 17	Ex17-F	CTCCCGCAATTCCTAGAAAATA
	Ex17-R	TTTGAGTGTTTTTCATTCTGCA
	Ex17-P	FAM-TGAGTTTGTGTGTGAACGGACACT-BHQ1
Exon 18	Ex18-F	TACATACAGCAGAAGAACGTGCTCT
	Ex18-R	GTCATTCTTCCTGTGCTCTTTTGT
	Ex18-P	HEX-CGCTGACCTCTCTATCTCCGTGA
Exon 19	Ex19-F	TTCTGTCCTGGGATTCTCTTG
	Ex19-R	GAGGAGATGTGGTCAATGGA
	Ex19-P	FAM-CTCGCTTTGGACCTTGGTGG-BHQ1
Exon 20	Ex20-F	TGGGGTTCTCCCAGGCTCT
	Ex20-R	GGGCTAGAAATCTGTTGCTATGG
	Ex20-P	HEX-CCTTCACCAACATGCCCACAG
Exon 21	Ex21-F	GGGTGAATGATGAAAGCTCCT
	Ex21-R	AACTGGAATGGATGGTACAGC
	Ex21-P	FAM-TCACCACAGAAGCACCACACA-BHQ1
Exon 22	Ex22-F	CAAAAGGACCCCATATAGCA
	Ex22-R	CAGAGGACAATGGCTTCCAT
	Ex22-P	FAM-CAGGTACATGCAGGCACCTTACC-BHQ1
Exon 23	Ex23-F	AGGCACCTGTGGTGACC
	Ex23-R	GGTAGGTGTCCAGCTCCTG
	Ex23-P	HEX-CTACACTGTCCAACACCCACTCTC-BHQ2

for one sample, simultaneously monitoring the usability of each separate reaction via estimation of the reference gene concentration. For this purpose, *ALB* (4q13.3) and *RPP30* (10q23.31) were chosen, which are common reference genes widely used as robust references for quantification of human genomic DNA [18–21].

Amplification of 4 targets in each group was multiplexed based on the amplitude of fluorescence. Specifically, four independent targets (three *BRCA1* exons and reference gene or two *BRCA1* exons and two reference genes) were amplified simultaneously in one reaction, where two probes for two targets have similar fluorophore (e.g., FAM or HEX). Simultaneously, two probes with the same fluorophore were at different concentrations in the reaction mix, resulting in different amplitudes of end-point fluorescence for each target. Therefore, in theory, up to 16 clusters of droplets can be formed, each containing from 0 to all 4 different templates, providing simultaneous quantification of four targets (Fig. 1). Comparison of concentrations between each exon and reference genes underlies the decision about the possible presence of aberration in the *BRCA1* gene.

Primers and probes for each exon and reference genes were designed using OligoAnalyzer software (standard conditions: 900 nM primers, 250 nM hydrolyzing probes, 50 mM Na^+, 3 mM Mg^{2+}, 0.4 µM dNTPs) according to the following standards. The annealing temperature was set at the range of 60–62 °C for primers and 65–67 °C for probes, respectively, with the absence of G at the 5′ terminus of hydrolyzing probe. The lengths of primers and probes were in the range of 18–27 bases, and Gibbs free energy ΔG of self-dimers was more than or equal to − 8 kcal/mol. The length of the resulting amplicon was found in the range of 70–100 bp. Each set of primers and probe was tested with real-time PCR to preliminarily confirm primers/probe suitability for ddPCR. All the designed primers/probes were determined as acceptable for ddPCR, showing threefold increase in fluorescence amplitude after PCR and Cq less than 28. After the test in real-time PCR, 8 tetraplexes consisting of primers/probe for 3–2 *BRCA1* exons and 1–2 reference gene (*ALB*, *RPP30*) were prepared and tested in ddPCR.

The first step of our multiplex ddPCR method optimization was obtaining appropriate clusterization of different

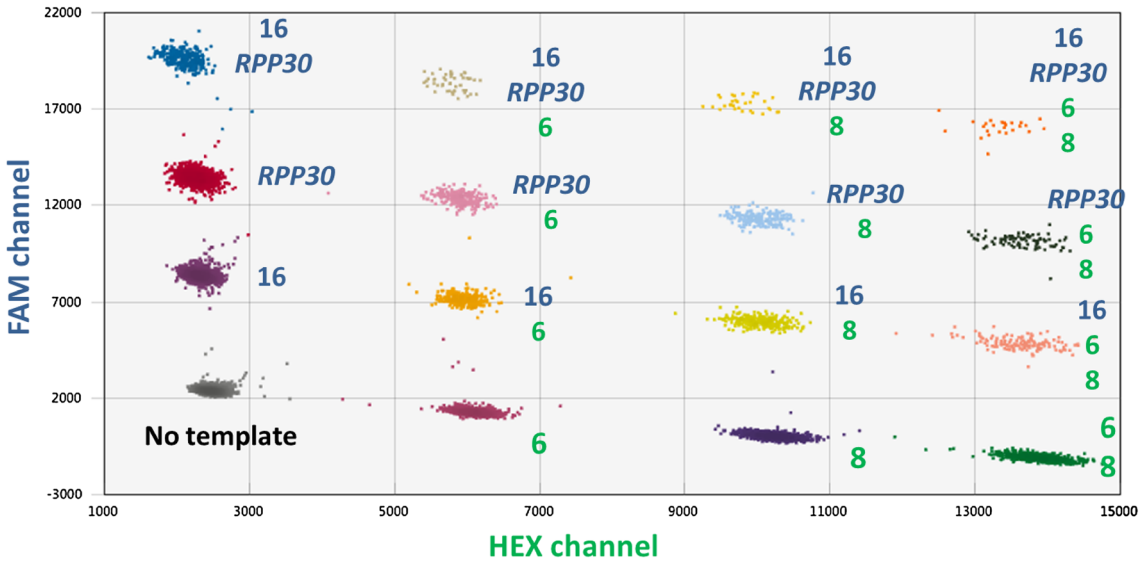

Fig. 1 2D representation of multiplex ddPCR reaction results. Fluorescence amplitude at channels FAM and HEX are plotted at *Y*- and at *X*-axis, respectively. Corresponding targets are designated for each cluster. Blue and green numbers denote the numbers of exons that are mixed in the multiplex

type droplets. Specifically, clusters should be located orthogonally; neighbor clusters should be separated by 2000–3000 RFU; no "rain" (fraction of droplets between two clusters) should be observed. Primers for amplification of *RPP30* appeared to be an obstacle for proper clusterization of exons 2 and 3 (Fig. 2a, b), decreasing amplitude of end-point fluorescence for the exon 3. On the contrary, no influence of primers on *ALB* was detected letting us see a clear difference between clusters. Therefore, to overcome the emerged obstacle with clusterization, reference gene in multiplex with exons 2, 3, 15 was changed from initially chosen *RPP30* to *ALB*. The same effect on amplification was noted for multiplex with exons 20, 22, 24, where primers for exon 24 impeded proper clusterization of exons 20 and 22 (Fig. 2c, d). The problem was solved via the redesign of primers set for exon 24; a new set of primers was just relocated regarding the first one. Sequences and concentrations of primers and probes, and the composition of multiplexes that were used in further work are listed in Tables 1 and 2.

Comparison of the presence and stability of homo- and hetero-oligonucleotide dimers with apparently deteriorated clusterization gave contradictory results (data not shown). All calculations were done using OligoAnalyzer software (Integrated DNA Technologies, Inc., USA) under previously specified standard conditions. *RPP30* primers form strong heterodimers with oligonucleotides for amplification of exons 2 and 3 that could give a glimpse to a reason of inconsistent clusterization in a multiplex of exons 2, 3, and *RPP30*. However, the first primer set for exon 24, being deleterious for cluster formation, forms less stable heterodimers with oligonucleotides for amplification of exons 20 and 22 than the second primer set. Thus, we could not formulate clear rules for multiplex ddPCR primer design just now.

After the optimization step, we developed the workflow for the analysis of the data obtained. Specifically, the ratio between mean concentration of each *BRCA1* exon and the mean concentration of each reference gene was calculated (see Materials and Methods section). For the samples studied, the mean ratio between *BRCA1* exons was 1.05 with a standard derivation of 0.093 and coefficient of variation of 0.088. Therefore, the difference from the mean ratio by more than two SD (~20%) was a cut-off for the positive decision concerning the deletion or insertion of a particular exon.

Influence of DNA concentration

The amount of template DNA loaded into a single reaction has a direct impact on the sensitivity of testing. Thus, miniscule DNA concentration, e.g., single template molecules, could lead to false results. Thus, the presence of different exons becomes stochastic in close to the limit of quantification amount of template.

To investigate the influence of DNA concentration on ddPCR sensitivity and to find an optimal range for quantification, we performed a titration of template in the range of 40–25,000 genome-equivalent per reaction. The highest value of the range was chosen based on the assumption about the most accurate measurement of DNA. According to the manufacturer's manual, in the used ddPCR system, the highest precision is achieved, when half of the droplets are positive, carrying DNA template. From the overall amount of 20,000 droplets, it gives 10,000 positive partitions or 10,000

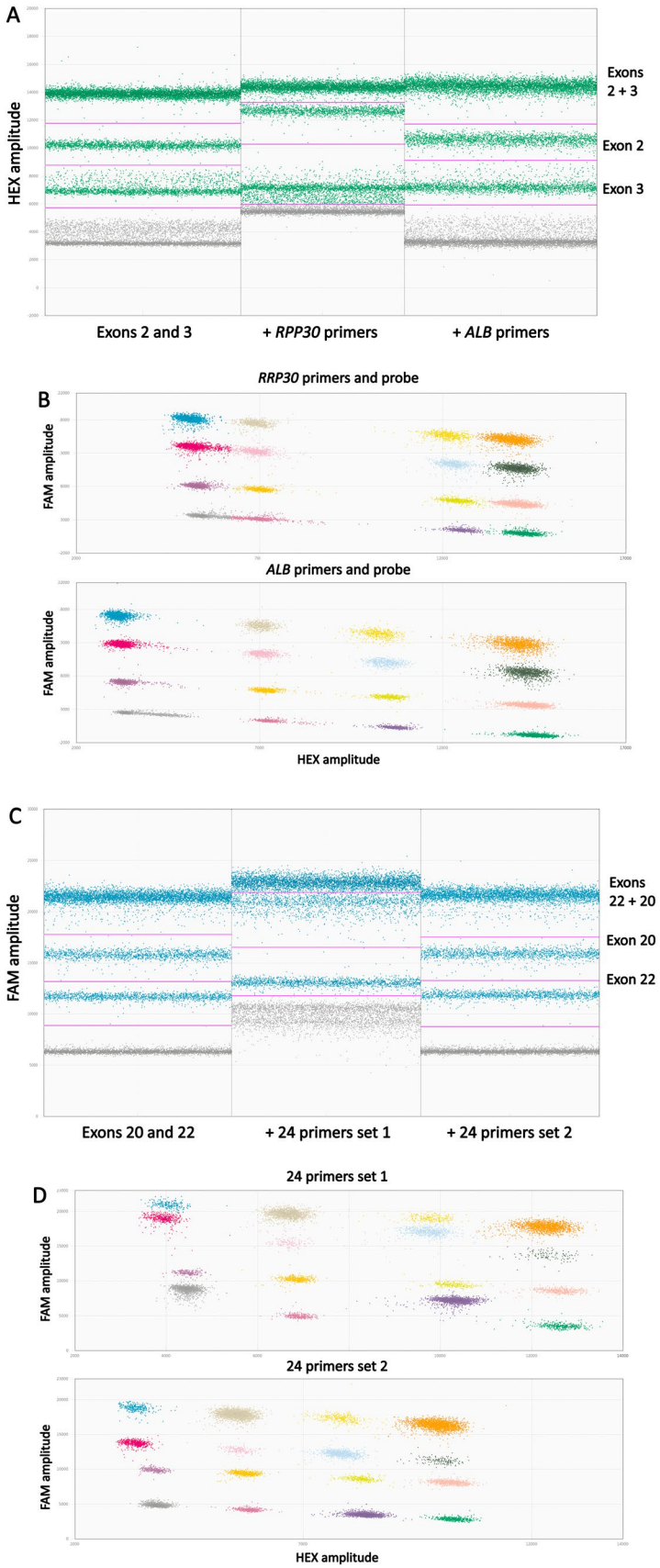

Fig. 2 Clusterization of optimized multiplexes. **a**, **b** multiplex of exons 2, 3, 15; **c**, **d** multiplex of exons 20, 22, 24. **a**, **c** 1D representation, where *Y*-axis represents fluorescence amplitude after reaction, *X*-axis shows samples. **b**, **d** 2D graphs of clusterization, fluorescence amplitude at channels FAM and HEX, is plotted at *Y*- and at *X*-axis, respectively

Table 2 Composition of primers and probes mixes for ddPCR

Mix	Target	Primers concentrations (μM)	Probe concentration (μM)	Relative fluorescence
1	Exon 1	9	2.5	FAM low
	Exon 4–5	18	5	FAM high
	Exon 14	9	2.5	HEX low
	RPP30	18	5	HEX high
2	Exon 15	9	2.5	FAM low
	ALB	18	5	FAM high
	Exon 3	9	2.5	HEX low
	Exon 2	18	5	HEX high
3	Exon 16	9	2.5	FAM low
	RPP30	18	5	FAM high
	Exon 6	9	2.5	HEX low
	Exon 8	18	5	HEX high
4	Exon 7	9	2.5	FAM low
	Exon 9	18	5	FAM high
	Exon 17	9	2.5	HEX low
	RPP30	18	5	HEX high
5	Exon 10	9	2.5	FAM low
	Exon 12	18	5	FAM high
	Exon 19	9	2.5	HEX low
	ALB	18	5	HEX high
6	Exon 18	9	2.5	FAM low
	ALB	18	5	FAM high
	Exon 11	9	2.5	HEX low
	Exon 13	18	5	HEX high
7	Exon 21	9	2.5	FAM low
	ALB	18	5	FAM high
	Exon 23	9	2.5	HEX low
	RPP30	18	5	HEX high
8	Exon 22	9	2.5	FAM low
	Exon 20	18	5	FAM high
	Exon 24	9	2.5	HEX low
	RPP30	18	5	HEX high

template molecules per reaction. Here we surpassed that number in 2.5-folds for validation of the developed ddPCR assay. In turn, the lowest template amount, 40 copies of template per reaction, is close to a theoretical limit of quantification with a strong influence of stochastic variations.

Control DNA sample with wild-type *BRCA1* and a clinical sample with a deletion of exons 1–2 were used as a template for the experiment (Fig. 3a, b). *BRCA1* exons and reference genes (*RPP30*, *ALB*) concentrations and, therefore, deletion can be precisely defined up to 200 copies of template per reaction. When the latter DNA amount was loaded in reaction, standard derivation increased from 0.093 for 1000 copies per reaction to 0.22 for 200 copies per reaction, making it difficult to assess the results and identify possible deletion. Consequently, reduced template amount (e.g., 40 copies per reaction) resulted in the failure to detect deletion due to the high dispersion between concentrations of different exons, when SD of ratio for different exons is in the range of 0.063–1.02.

The results obtained indicated that designed ddPCR assay can be used in a broad range of template concentration from at least 25,000 to 1000 copies of template per reaction.

Validation of ddPCR assay

To examine the ability of designed 8 multiplexes set to detect rearrangements in the *BRCA1* gene, 24 clinical specimens were tested; genomic DNA was purified from whole blood. Presence of *BRCA1* rearrangements in specimens was confirmed using commercial MLPA-based kits. Among the 24 tested samples, 9 harbored deletion and 1 had duplication.

The results of the ddPCR assay are presented in Fig. 4. All samples tested by MLPA as wild-type *BRCA1* (13) were also identified by ddPCR as wild-type *BRCA1* likewise. Digital PCR assay also demonstrated 100% concordance with MLPA in specimens with *BRCA1* rearrangements; no disparate results in determining the type and borders of rearrangement were observed.

In spite of clear discrimination between wild-type *BRCA1* and deletion/duplication variants, miniscule but omnipresent difference between *BRCA1* exons concentrations was found (Supplementary Figure 1). In each studied sample, the concentration of certain *BRCA1* exons was higher or lower than the mean value between all exons, leading to a discordance in *BRCA1* exon/reference gene ratio. We tried to explain the variability of exon representation by structure and content of amplicons produced by PCR. For each amplified region we compared GC content [22, 23], AG content (content of purines), amplicon length, number of non-target regions to which primers can hybridize, and number of four-mers in the amplicon [24] that have reverse-complement sequences in the same amplicon. For two amplicons with the lowest median representation (exons 19 and 23), we suggested two possible reasons for exon concentration lessening. Representation of exons 19 and 23 amplicons could vary due to the significant difference of the purine content in two strands and the high number of non-target hybridization sites for the primers, respectively, that could lead to less efficient single DNA molecule amplification in droplet.

Discussion

The main method used to detect large rearrangements in the *BRCA1* gene in clinics is MLPA. Recently, several new effective methods to search for such type of mutations in the *BRCA1* were developed [17, 25, 26]. They are based on

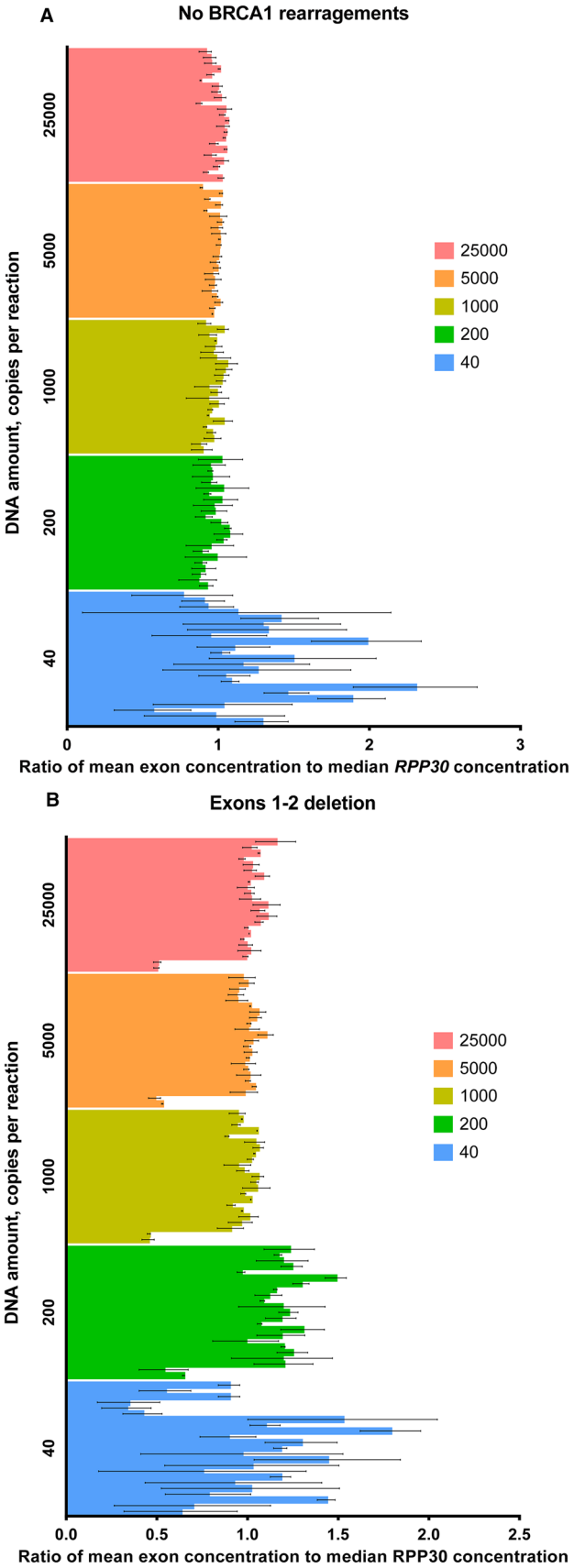

Fig. 3 Influence of DNA amount on ddPCR performance. **a** wild-type *BRCA1* specimen, **b** specimen with deletion of *BRCA1* exons 1–2. Each specimen was analyzed in duplicates. *X*-axis represents the ratio of mean exon concentration to median *RPP30* concentration and *Y*-axis shows the amount of loaded DNA template in copies per reaction. Exons are ordered bottom-upwards. Whiskers designate standard deviation in two experiments

high-resolution melting analysis, ddPCR, and NGS. For all techniques concerned, the results obtained were confirmed by MLPA, but none has been implemented into practice yet. It may be due to the high cost of the approach applied, as in [17] or to the dependence on the quality of analyzed DNA. Droplet digital PCR allows calculating the exact number of specific DNA molecules, but its relatively high cost of straightforward usage needs to perform a large number of individual reactions corresponding to the number of exons studied. Multiplexing several PCRs into one reaction could reduce labor and reagent costs.

Two approaches can be applied for multiplexing of ddPCR [27]. The first strategy (ratio-based) is based on blending for one target two hydrolysis probes with different fluorophores, resulting in droplets cluster with simultaneous fluorescence at both channels. This approach has been used for simultaneous quantification of three targets, or detection of several somatic mutations [28, 29]. The principle of the second strategy (amplitude-based) is performing ddPCR with different concentrations of probes for various targets, permitting the targets to be identified by fluorescence amplitude of corresponding clusters. Recently, amplitude-based multiplex ddPCR has been applied for the detection of transgene plants [30] or somatic mutations [31]. The amplitude-based approach in the case of tetraplexes gives easily recognizable 16 clusters, located rectangularly. According to our past experience with multiplex ddPCR, such type of multiplexes can be plainly designed and optimized with little or no efforts. At the same time, the ratio-based approach requires two probes with different fluorophores for one target, and adds more cost for analysis setup. Here we employed amplitude-based strategy due to its apparent simplicity and clarity of results.

In the present work, we encountered insufficient clusterization in two multiplexes, which was amended by the change of primers for one of the targets. In quantitative multiplex PCR, primers and probes can interact with each other, affecting the efficacy of amplification of the specific target(s). Little is known as how it could be applied to the design of multiplex digital PCR. Commonly used concentrations of primers and probes in ddPCR are higher than in qPCR [32], suggesting a higher risk for spurious dimeric or more complex duplex structures to be formed. In qPCR, those structures could diminish amplification efficacy,

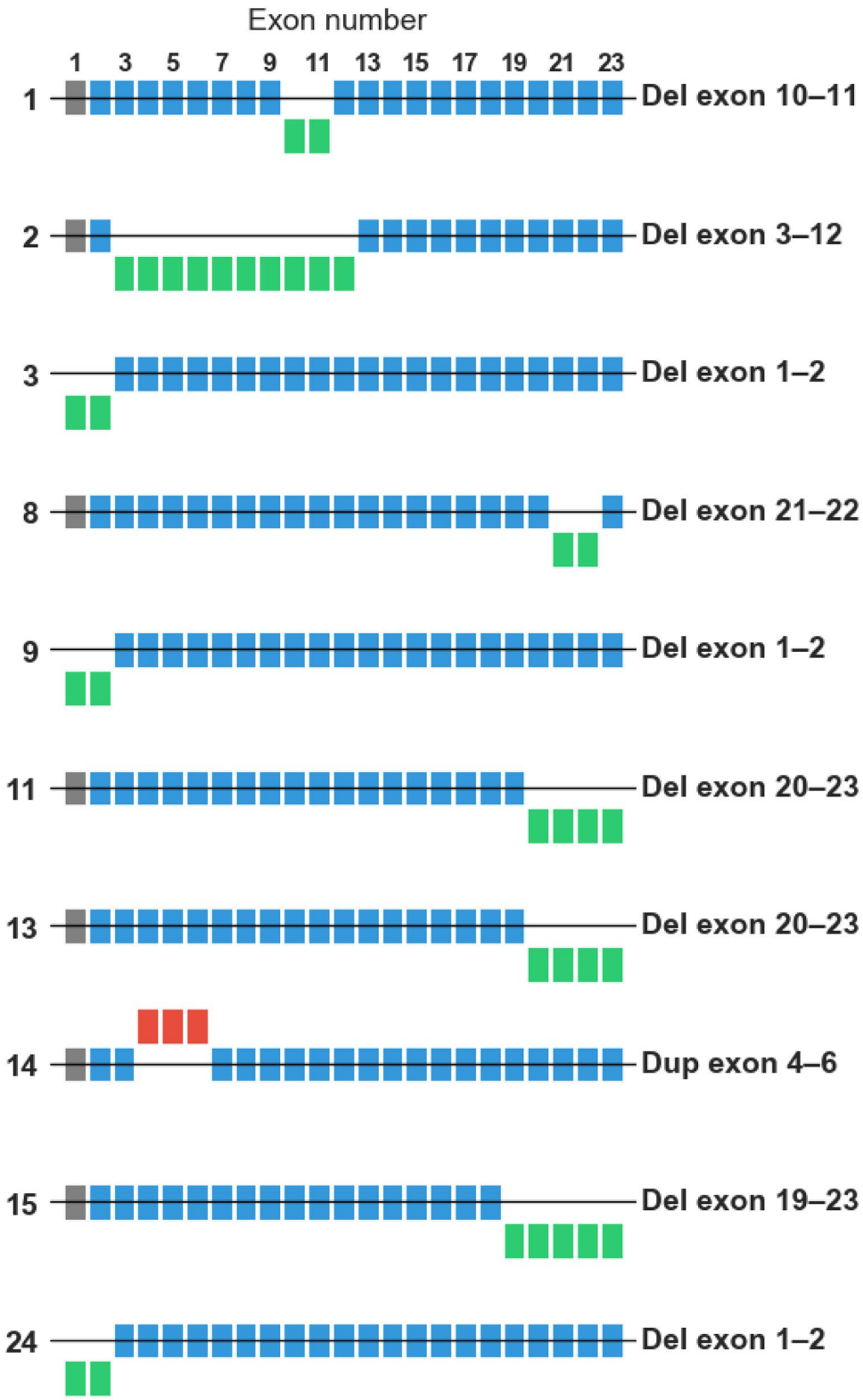

Fig. 4 Schematic representation of *BRCA1* rearrangements spectrum in tested specimens. All specimens were analyzed using both commercial MLPA kits and in-house ddPCR assay; only samples with *BRCA1* rearrangements are depicted. Each exon is shown as a colored box (in a non-scale manner), where blue boxes show the absence of rearrangement, green boxes reflect deletion, and red ones duplication. Specimen number, exon location, and rearrangement type are given at the left, upper, and right sides, respectively

undermining quantification. For ddPCR, no holistic insight with a mathematical model of oligonucleotides interactions has yet been developed. Intriguingly, no direct correlation between the calculated stability of heterodimers and incoherent clusterization was found, indicating the presence of more complex processes and factors influencing clusterization in digital PCR.

A similar problem appeared during the validation of developed ddPCR assay when we observed a stable difference between *BRCA1* exons concentrations. Our attempts to disclose the reason for detected discordance have not yielded clear results. Only for two exons with a decreased representation, a correlation with a high purine content of the corresponding amplicons was found. Other studied variables (GC content, amplicon length, number of non-target regions to which primers can hybridize, number of four-mers in the amplicon that have reverse-complement sequences in the same amplicon) showed no correlation with increased or decreased *BRCA1* exon representation. Taken together, insufficient clusterization and variation in exons concentration emphasize the need to investigate interactions of oligonucleotides in ddPCR conditions.

BRCA1 gene consists of 23 exons; each exon can be deleted or duplicated, necessitating simultaneous quantification of all exons. While MLPA is a common method for *BRCA1* rearrangement testing, allowing time- and cost-effecting detection of deletions and insertions in the gene, a number of limitations can hinder the analysis. Thus, MLPA is more sensitive to contaminants than PCR, requires several nanograms (several thousands of genome-equivalents) of DNA, demands several references for proper analyses, and provides information only about the relative presence of loci. Digital PCR is believed to be more robust to inhibitors, needs much less DNA template, and yields information about absolute DNA concentration. Our results indicate that multiplex digital PCR can be successfully used for detecting *BRCA1* rearrangements, providing results in concordance with MLPA-based reference method.

Another competitive technology for CNV detection is NGS with a clear advantage of the simultaneous identification of point mutations and CNV [26]. This technology is becoming common in clinical practice. However, as in the case of ddPCR, no NGS-based assays for detecting large rearrangements in *BRCA1* and *BRCA2* genes have been proved and registered for clinical applications. For small laboratories with a low sample flow, ddPCR can be useful as an opportunity to test one to several samples per run instead of collecting many samples for one NGS-run.

Regardless of high accuracy and robustness, the developed ddPCR assay has several limitations. If handled incautiously, ddPCR is prone to contamination by amplicons, produced during amplification in a massive amount. Contamination could lead to a disturbance in obtained results and requires considerable effort to eliminate it. Therefore, workplace for ddPCR should be organized in one-way movement of samples; any possible contacts of ddPCR reagents with processed samples after amplification should be prevented.

Cost of equipment and reagents for ddPCR is also higher than for MLPA. However, ddPCR can be used in clinical diagnostics in many other applications that require high sensitivity and/or high precision of analysis, specifically, detection of traces of tumor or fetus DNA in human blood plasma. As far as we can see, in the future, most clinical laboratories are likely to have the appropriate staff and the ability to use ddPCR technology, as can be seen in the case of NGS technologies.

Analysis of FFPE-derived DNA samples also could be challenging. During FFPE samples preparation, DNA is exposed to various chemicals resulting in fragmentation and multiple modifications of nucleotides. Amplification efficacy of fragmented and modified FFPE DNA is relatively low due to polymerase stalling at the sites of modifications. In the case of ddPCR, it results in droplets with intermediate fluorescence, e.g., "raining", which hinders accurate quantification of DNA. In the present work, we focused on blood-derived DNA samples to confirm the use of multiplex ddPCR for *BRCA1* testing. Applicability of the developed assay for FFPE-derived DNA samples should be proved in future studies.

Here we report the first multiplex digital PCR assay for the detection of *BRCA1* rearrangements. The developed assay provides results in accordance with MLPA and can be used for routine clinical screening.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All patients signed an informed written consent to participate and to have their biological specimens analyzed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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