Evaluation of two new highly multiplexed PCR assays as an alternative to next-generation sequencing for IDH1/2 mutation detection

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IDH1 and IDH2 somatic mutations have been identified in solid tumors and blood malignancies. The development of inhibitors of mutant IDH1 and IDH2 in the past few years has prompted the development of a fast and sensitive assay to detect IDH1R132, IDH2R140 and IDH2R172 mutations to identify patients eligible for these targeted therapies. This study aimed to compare two new multiplexed PCR assays – an automated quantitative PCR (qPCR) on the PGX platform and a droplet digital PCR (ddPCR) with next-generation sequencing (NGS) for IDH1/2 mutation detection. These assays were evaluated on 102 DNA extracted from patient peripheral blood, bone marrow and formalin-fixed paraffin-embedded tissue samples with mutation allelic frequency ranging from 0.6% to 45.6%. The ddPCR assay had better analytical performances than the PGX assay with 100% specificity, 100% sensitivity and a detection limit down to 0.5% on IDH1R132, IDH2R140 and IDH2R172 codons, and a high correlation with NGS results. Therefore, the new highly multiplexed ddPCR is a fast and cost-effective assay that meets most clinical needs to identify and follow cancer patients in the era of anti-IDH1/2-targeted therapies.

1. Introduction

Isocitrate dehydrogenase enzymes 1 (IDH1) and 2 (IDH2) form homodimers to catalyze the reversible NAD+ dependent oxidative decarboxylation of isocitrate to α-ketoglutarate (α-KG) [1]. IDH1 is localized in peroxisomes and the cytosol, whereas IDH2 is located in the mitochondria [2]. Since the discovery of a somatic IDH1 mutation in colorectal cancer patients [3], other mutations of IDH1 and IDH2 have been identified in many other cancers.

Abbreviations
2HG, (D)-2-Hydroxyglutarate; α-KG, α-ketoglutarate; AITL, angioimmunoblastic T-cell lymphoma; AML, acute myeloid leukemia; CCA, cholangiocarcinomas; CRC, colorectal carcinoma; DNA, deoxyribonucleic acid; ddPCR, droplet digital PCR; FFPE, formalin-fixed paraffin-embedded; GB, glioblastomas; IDH1&2, Isocitrate Dehydrogenase 1&2; IGV, integrative genomics viewer; LOB, limit of blank; LOD, limit of detection; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms; NGS, next-generation sequencing; qPCR, quantitative PCR; ROC, receiving operating characteristics; SNVs, single-nucleotide variants; VAF, variant allele frequency.
cancers such as secondary glioblastoma and glioma [4–6] (~ 80%), chondrosarcoma (50–80%) [5,7], thyroid carcinoma (16%) [5,8] intrahepatic cholangiocarcinoma (10–20%) [5,9], and in other types of solid tumors at lower frequencies [5,10,11]. IDH1/2 mutations are also present in hematological malignancies, mainly in acute myeloid leukemia (AML, ~ 20%) [5,12], but also in myeloproliferative neoplasms (MPN, ~ 1–4%) [13], in myelodysplastic syndromes (MDS, ~ 5%) [12] and angioimmunoblastic T-cell lymphomas (AITL, ~ 20–30%) [5,14]. IDH1 and two mutations are missense mutations located on three hotspot codons: IDH1R132, IDH2R140, and IDH2R172. These amino acids are localized within the catalytic domain of the enzymes. Their mutation results in the activation of a neomorphic activity that converts α-KG into (D)-2-Hydroxyglutarate (2HG). The latter is an oncometabolite involved in DNA and histone hypermethylation leading to gene transcription deregulation and cell differentiation blockage [15]. The frequency of mutated codons varies across malignancies [5]. IDH1R132 and IDH2R172 are found in solid tumors, while IDH2R140 is rarely mutated. On the contrary, all three IDH1/2 codons can be altered in myeloid malignancies, and only IDH2R172 is affected in AITL [16,17].

In the past few years, anti-IDH1 or IDH2 targeted therapies have been actively evaluated alone or in association with 5-azacytidine in refractory, relapsed or newly diagnosed AML [18–20]. Several clinical trials are also evaluating these targeted therapies on advanced or refractory cholangiocarcinoma [21,22], on advanced-stage chondrosarcoma [23], or glioma [24]. The Food and Drug Administration (FDA) recently approvedivosidenib to treat newly diagnosed or relapsed AML [25], and for locally advanced or metastatic cholangiocarcinoma.

Given all these therapeutic developments, it has become increasingly necessary to genotype IDH1 and IDH2 hotspot codons for these malignancies. We and others have developed multiple PCR, immunohistochemistry [26–29], mass spectrometry [7] or Sanger sequencing assays. However, most of these methods were not designed to detect mutations on all three codons with high specificity and sensitivity. For these reasons, next-generation sequencing (NGS) has become the standard for IDH1/2 mutation detection and quantification in many laboratories analyzing both solid tumors and hematological malignancies. However, NGS remains a cost and time-consuming method that hardly meets the clinical need of fast result release compared to PCR assays. The objective of our study was to compare two new multiplexed PCR assays – an automated qPCR and a droplet digital PCR (ddPCR) as an alternative to NGS for IDH1/2 mutation detection.

2. Materials and methods

2.1. Samples

DNA samples from 102 patients with acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), angioimmunoblastic T-cell lymphoma (AITL), cholangiocarcinomas (CCA), glioblastomas (GB), melanoma, colorectal carcinoma (CRC) or commercial control (HD829, Myeloid DNA Reference Standard, Horizon) were collected for this study and were assessed using NGS. This study was conducted as part of the care in Henri Mondor University Hospital in compliance with French regulations and approved (no. 2020-083) by the Henri Mondor Institutional Review Board (No. 00011558). The study methodologies conformed to the standards set by the Declaration of Helsinki. All patient data were anonymized and deidentified before analysis. A letter of nonobjection explaining the purpose of this study was sent to each patient. Sixty DNA samples were assessed using Easy-PGX-ready IDH1/2 kit. Ninety-nine DNA samples were evaluated using ddPCR (Table 1, Fig. 1, and Table S1).

2.2. DNA extraction

DNA extractions were performed from formalin-fixed, paraffin-embedded (FFPE) tissue sections (usually seven sections, 5 μm thick) using the Maxwell RSC DNA FFPE Kit IVD (Promega, Charbonnières-Les-Bains, France) as previously described [30–32], or from bone marrow and whole blood samples using QIAsymphony DSP DNA midi kit® and Qiaphoney extraction platform (Qiagen, Courtaboeuf, France) according to the manufacturer’s instructions. DNAs were quantified using a Qubit fluorimeter with the Qubit dsDNA HS Array Kit (ThermoFisher Scientific, Ilkirch, France).

2.3. IDH1/2 genotyping by next-generation sequencing

DNA samples were sequenced using a multiplex PCR-based enrichment (Ampliseq custom panel, ThermoFisher Scientific) or a custom hybridization capture-based target enrichment (SureSelect QXT Library Prep Kit, Agilent, Courtaboeuf, France).

As previously described, [28], 10 ng of DNA was amplified using an Ampliseq custom panel (Thermo-Fisher Scientific), a multiplex PCR-based library-preparation method by which many regions (70–
150 bp), including those coding for IDH1 and IDH2, were amplified. Amplicons were then digested, bar-coded and amplified using the Ion Ampliseq kit for Chef DL8 and Ion Xpress barcode adapter kit (ThermoFisher Scientific), according to the manufacturer’s instructions. After DNA library preparation and quantification, 25 pm of each library was multiplexed and clonally amplified on ion-sphere particles (ISP) by emulsion PCR performed on Ion Chef (ThermoFisher Scientific), according to the manufacturer’s instructions. The ISP templates were loaded onto an Ion-510 or 520 chip and sequenced on an S5 sequencer with the Ion 510™ & Ion 520™ Kit–Chef, according to the manufacturer’s instructions. Run performance was assessed and data analyzed with the TORRENT SUITE Software v.5.10.0 (ThermoFisher Scientific). Sequencing data were analyzed using two different pipelines: IONREPORTER (ThermoFisher Scientific) and SeqPilot (JSI Medical Systems, Ettenheim, Germany). Single-nucleotide variants (SNV) were visualized using the Variant Caller plug-in version 5.10.0.18 with low stringency settings (threshold: 2%).

For the QXT assay, libraries were prepared according to the manufacturer’s instructions, except that 70 ng of input DNA was used to prepare all libraries. 85 kb regions, including hotspots or all exons of 38 genes, were captured and sequenced with an Illumina v3 2 × 300 bp chip on MiSeq (Illumina®, San Diego, CA, USA). Demultiplexing and adapter trimming was performed with MiSeq reporter. Fastq files were trimmed with SICKLE (v1.33) to remove low-quality bases and then aligned on hg19 reference genome with bwa-mem (v0.7.15). Realignment and base score recalibration was performed with gatk (v3.8.0) according to the Broad Institute guidelines. SNVs and small indels were called with VARSCAN2 (v2.4.3) and HAPLOTYPE CALLER (v3.8.0) on the whole captured genomic positions. On specific hot spots (including IDH1R132, IDH2R140, and IDH2R172), a second calling was performed with VARSCAN2 using the following parameters: minimum supporting reads = 5; minimum base quality at a position to count a read = 13 and minimum variant allele frequency (VAF) threshold = 0.005 and Ignore variants with > 90% support on one strand = No. The Integrative Genomics Viewer (IGV v 5.01; Broad Institute, Cambridge, MA, USA) was used for visual low VAF mutation curation.
2.4. IDH1/2 genotyping by EASYPGX

DNA samples were assessed for IDH1 (codons 105 and 132) and IDH2 mutations (codons 140 and 172) using EASYPGX® ready IDH1/2 kit (Diatech Pharmacogenetics, Jesi, Italy; Table S2).

Twenty-five nanogram of DNA extracted from blood or FFPE is added for each position, and PCR is achieved according to the manufacturer’s instructions. Data were analyzed using EASYPGX software.

2.5. IDH1/2 genotyping by droplet digital PCR

An allele-specific droplet digital PCR assay (Biorad®, Marnes-la-Coquette, France) designed to detect six mutations on IDH1R132 codon (dHsaEXD61571942), four mutations on IDH2R140 codon (dHsaEXD35841715) and five mutations on IDH2R172 codon (dHsaEXD10111488) was evaluated (Table S3). Briefly, 1.1 µL of DNA at 30 or 50 ng·µL⁻¹ was added to each master mix and then automatically encapsulated in 15,000 to 20,000 droplets. Fluorescent droplets were then quantified using the QX200 droplet reader (Biorad®). Each run was analyzed using QUANTASOFT software (version 1.7.4; Biorad®). Positivity thresholds were set for each sample as the signal to background ratios depended on the codon and the type of mutation detected. When only double-positive droplets (for mutant and wild-type allele) were present, usually at a limited number (< 5 droplets) and displaying an intermediate fluorescence intensity, these were considered as negative artifactual events. In contrast, single positive droplets were always considered as positive events. Thresholds were thus adjusted accordingly. Following a temperature gradient PCR experiment, the annealing temperature was set at 55 °C as it optimally separated positive droplets from the fluorescence background signal. The number of droplets was converted by QUANTASOFT into copies of mutant or wild-type alleles for each codon using Poisson statistics. Results were expressed using the fractional abundance provided by QUANTASOFT® software (Biorad®) as follows: 

\[ \%VAF = \frac{100 \times IDH_{\text{mutant copies}}}{IDH_{\text{mutant copies}} + IDH_{\text{wildtype copies}}} \]

The following formula was applied to evaluate the limit of blank (LOB): 

\[ \text{LOB} = \text{meanblank} + 1.645 \times SD_{\text{blank}} \]

with SDblank corresponding to the standard deviation (SD) of background allelic frequencies measured on all wild type codons evaluated in this study (Table 1; IDH1R132, n = 73 samples, IDH2R140, n = 82 samples, and IDH2R172, n = 75 samples). The limit of detection (LOD) was estimated by two different methods. For LOD1, 30 ng of seven mutated DNA samples including sample 9 (IDH1R132S, 32.8%), sample 3 (IDH1R132C, 31.5%), sample 12 (IDH1R132G, 33.9%), sample 33 (IDH2R140Q, 32.8%), sample 27 (IDH2R140W, 43.8%), sample 49 (IDH2R172K, 31.6%) and sample 57 (IDH2R172G, 15.0%) were serially diluted at a 1 : 4 ratio in a wild-type (WT) DNA (sample 93) to obtain standards with VAF ranging from 44.3% to 0.03% (Table S1). The following formula was used: 

\[ \text{LOD1} = \text{LOB} + 1.645 \times SD_{\text{low}} \]

with SDlow
corresponding to the average standard deviation of variant allelic frequencies found for the last two dilutions (VAF at ~0.5% and VAF at ~0.125%) [33]. For LOD₂, the following formula was applied: \[\text{LOD}_2 = \text{MeanBlank} + 5\times\text{SDblank}.\]

### 2.6. Statistical analysis and diagnostic values

All data were collected using EXCEL software. GRAFPAD PRISM software version 6.0 and MEDCALC STATISTICAL software version 12.7.5 (Ostend, Belgium) were used to perform correlation tests, Bland–Altman graphs [34] and Receiving Operating Characteristics (ROC) curves. Accuracy, positive (+LR) and negative likelihood ratio (−LR) were calculated using formulas described in [35]. As no false positive result was detected, +LR values could not be determined (Table S4).

### 3. Results

#### 3.1. Validation set

In this study, the performances of two methods – EASYPGX (Diatech Pharmacogenetics) and a multiplexed droplet digital PCR (ddPCR) assay were compared to targeted NGS sequencing. We selected 102 DNAs initially sequenced by NGS to detect and quantify \(\text{IDH}1^{R132}, \text{IDH}2^{R140},\) and \(\text{IDH}2^{R172}\) mutations. The characteristics of all samples analyzed are summarized in Fig. 1, Table 1 and Table S1. Among these, 57 samples were extracted from peripheral blood (PB), or bone marrow (BM) derived from AML (n = 20) or MPN/MDS (n = 37) patients. Forty-four DNAs were extracted from formalin-fixed paraffin-embedded (FFPE) tissue samples from T-cell lymphoma (n = 26), cholangiocarcinoma (n = 13), glioblastoma (n = 3), colorectal carcinoma (n = 1) or melanoma (n = 1) patients, and one commercial DNA was also included. Among this DNA set, 36 were wild type (WT) on all three codons, and 66 DNAs were mutated according to NGS results. Twenty-four samples carried a single-nucleotide variant (SNV) on codon 132 (R132C/S/G/L/H), 17 on codon 140 (R140W/Q), and 23 on codon 172 (R172K/T/G/S/W). Two samples had concomitant R132 and R172 mutations: the commercial DNA (both with a variant allelic frequency at 5%) and one AML patient DNA with concomitant \(\text{IDH}1^{R132C}\) and \(\text{IDH}2^{R172K}\) mutations below the variant calling threshold but was visually confirmed in the bam file (Fig. S1A). Altogether, 68 mutations were detected (66 single mutations and two double mutations) with VAF ranging from 0.4% to 45.5%, and 76, 85, and 77 DNAs were considered WT on 132, 140, and 172 codons, respectively. To evaluate PGX and ddPCR assay performance, 60 samples were analyzed by both assays, 24 by ddPCR only and two by PGX only.

#### 3.2. PCR limit of blank and limit of detection

PGX limit of detection (LOD) was determined and provided by PGX supplier at 0.5% for \(\text{IDH}1^{R132S,R132G,R132L,R132I,R132V}\) and \(\text{IDH}2^{R140G,R140W,R140Q,R140W}\), 5% for \(\text{IDH}2^{R172K}\) and 1% for \(\text{IDH}1^{R172G,R172W,R172T,R172M,R172S}\) (Table S2). For ddPCR, the average background signal (meanblank) was evaluated on 73 WT samples for \(\text{IDH}1^{R132}, 82\) for \(\text{IDH}2^{R140}\), and 75 for \(\text{IDH}2^{R172}\) and found statistically different between FFPE samples and non-FFPE (blood/bone marrow) samples for \(\text{IDH}1^{R132}\) (unpaired t-test with Welch’s correction, \(P = 0.0003\)), \(\text{IDH}2^{R140}\) (unpaired t-test with Welch’s correction, \(P = 0.0001\)) and \(\text{IDH}2^{R172}\) codons (unpaired t-test with Welch’s correction, \(P = 0.0091\)). The limit of blank (LOB) was found at 0.29% for codon \(\text{IDH}1^{R132}\), 0.38% for codon \(\text{IDH}2^{R140}\) and 0.11% for codon \(\text{IDH}2^{R172}\) (Table 2). The LOB was further evaluated according to sample pre-treatment. The LOB were lower for non-FFPE samples (0.14% for codon \(\text{IDH}1^{R132}\), 0.14% for codon \(\text{IDH}2^{R140}\) and 0.02% for codon \(\text{IDH}2^{R172}\) as compared with FFPE samples.

| IDH1 R132 | IDH2 R140 | IDH2 R172 |
|-----------|-----------|-----------|
| MeanBlank (%) | 0.07 | 0.10 | 0.02 |
| MeanBlank non FFPE (%) | 0.02 | 0.02 | 0.004 |
| SDBlank (%) | 0.13 | 0.17 | 0.05 |
| SDBlank non FFPE (%) | 0.1 | 0.1 | 0 |
| SDBlank FFPE (%) | 0.2 | 0.2 | 0.1 |
| LOB (%) | 0.29 | 0.38 | 0.11 |
| LOB non FFPE (%) | 0.14 | 0.14 | 0.02 |
| LOB FFPE (%) | 0.44 | 0.51 | 0.18 |
| LOD1 (%) | 0.6 | 0.5 | 0.2 |
| LOD1 non FFPE (%) | 0.5 | 0.3 | 0.2 |
| LOD1 FFPE (%) | 0.8 | 0.7 | 0.3 |
| LOD1 (%) | 0.7 | 0.9 | 0.3 |
| LOD2 non FFPE (%) | 0.4 | 0.4 | 0.1 |
| LOD2 FFPE (%) | 1.0 | 1.2 | 0.5 |
(0.44% for codon \textit{IDH1R132}, 0.51% for codon \textit{IDH2R140}) and 0.18% for codon \textit{IDH2R172}). The limit of detection (LOD) was assessed by serial dilution of seven DNA samples carrying \textit{IDH1R132S}, \textit{IDH1R132C}, \textit{IDH1R132G}, \textit{IDH2R140Q}, \textit{IDH2R140W}, \textit{IDH2R172K}, and \textit{IDH2R172G} mutations mixed at a 1 : 4 ratio in a WT DNA to obtain standards with VAF ranging from 44.3% to 0.03% (Fig. 2). The coefficient of determination ($r^2$) ranged between 0.99 and 1 for the seven mutations analyzed, confirming the linearity of ddPCR on the entire range of VAF tested. However, for \textit{IDH1R132C}, \textit{IDH2R140W}, \textit{IDH2R172G}, and \textit{IDH2R172K} mutations, ddPCR quantification was not linear when the VAF was below 1%. For all the mutations tested, ddPCR detected mutations with VAF down to 0.03%. The LOD was first calculated on all samples by two

**Fig. 2.** ddPCR limit of detection. The seven graphs represent the ddPCR variant allelic frequency (VAF) in terms of theoretical VAF obtained by multiplying \textit{IDH1} (upper graphs) or \textit{IDH2} (middle and lower panel) mutant VAF of the undiluted DNA sample by the dilution ratio. Each dot represents the mean of VAF quantified by ddPCR in duplicates, and the black lines correspond to the best fitting log-log line. $r^2$, Coefficient of determination; ddPCR, Droplet digital PCR.
methods for each codon and ranged between 0.2% to 0.9% depending on the codon and the method of LOD calculation (Table 2 and Materials and methods). The LOD were also evaluated according to sample pre-treatment and were found different. To take into account the significant increase in the ddPCR background signal associated with FFPE samples the LOD were finally set on all three codons at 0.5% and 1.2% for blood/bone marrow and FFPE samples respectively. These values corresponded to the highest LOD identified among the three codons analyzed.

3.3. Sensitivity and specificity of PGX and ddPCR

All IDH1R132 mutations were found by PGX and ddPCR (Table 1), leading to a sensitivity and specificity for both assays at 100% and an area under the curve (AUC) at 1 (Fig. 3).

PGX detected 11/13 IDH2R140 mutations (Table 1 and Fig. 1). The two IDH2R140Q mutations found negative were detected at low allelic frequencies by NGS (4.8% and 6%), although the VAFs were above the PGX LOD (2%). All IDH2R140 (n = 17) were found mutated by ddPCR (Table 1). Overall, the sensitivity for IDH2R140 was 100% and 84.6% for ddPCR and PGX, respectively. AUC of ROC curves comparing both assays to NGS results was found at 1.0 and 0.923 for ddPCR and PGX, respectively (Fig. 3).

Only 5/14 IDH2R172 mutations (NGS VAF = 3% to 42%) were detected by PGX (LOD = 5% and 1%, Table S1) leading to a sensitivity at 35.7%. Conversely, ddPCR detected 22/22 mutations (NGS VAF = 0.6% to 45%). ROC curve AUCs were found at 1 and 0.679 for ddPCR and PGX, respectively.

There was no false-positive result for both assays on all three codons (Fig. 3).

Sensitivities (Se), specificities (Sp), negative and positive predictive values (NPV and PPV), likelihood ratios (LR) and accuracies were calculated for each codon for PGX and ddPCR, on the whole sample set and according to sample pre-treatment (Table S4). ddPCR was found equivalent and often superior to PGX for all these parameters. Notably, ddPCR negative LR were always found at 0, while it ranged between 0.15 and 0.67 for PGX.

Considering DNA samples tested on all three codons of IDH1 and IDH2, ddPCR showed much better analytical performances with a sensitivity, specificity, accuracy, negative and positive predictive values on all three codons at 100% compared with the PGX assay showing a lower sensitivity at 73.7%. Altogether, these results strengthen the analytical performance of ddPCR assay as compared with PGX.

3.4. ddPCR correlation with NGS

The comparison of VAF found by NGS and ddPCR showed an excellent correlation for all mutations: IDH1R132 (\(r^2 = 0.982, \ P < 0.0001\), linear regression), IDH2R140 (\(r^2 = 0.993; \ P < 0.0001\); linear regression) and IDH2R172 (\(r^2 = 0.959; \ P < 0.0001\), linear regression; Fig. 4A). Although ddPCR showed an excellent correlation with NGS on the three assays, \(r^2\) was lower for IDH1R132 and IDH2R172. 11/26 (42%) and 16/25 (64%) DNA samples carrying an IDH1R132 and IDH2R172 mutation were derived from FFPE samples. We, therefore, evaluated the correlation between ddPCR and NGS according to sample processing before DNA
extraction. DNA samples derived from blood/marrow or FFPE samples showed a similar correlation with NGS results (for \textit{IDH1R132}, \(r^2 = 0.98\) and \(r^2 = 0.95\) respectively; data not shown). Taken together, these results showed an excellent correlation between VAF obtained by NGS and ddPCR, including low VAF (under 1%) on peripheral blood, bone marrow, or FFPE samples.

The Bland and Altman diagrams did not identify a significant bias of VAF measured by ddPCR compared with NGS on \textit{IDH1R132}, \textit{IDH2R140}, and \textit{IDH2R172} codons (mean bias = -0.7, -0.6 and 0.1 respectively), confirming a good agreement between both assays (Fig. 4B).

**4. Discussion**

The detection of \textit{IDH1R132} and \textit{IDH2R140/R172} mutations is helpful information to guide the diagnosis of several malignancies such as glioblastomas [4], gliomas [6], AITL [17], MDS [36], or AML [37] and identify patients eligible for anti-IDH1 and/or anti-IDH2 targeted therapies. Given the running time of NGS analysis, the implementation of another rapid, sensitive and specific assay would be a valuable improvement in the molecular characterization of cancer patients. In this study, two new multiplexed PCR methods were compared to NGS. The ddPCR assay was an excellent alternative to NGS for detecting and quantifying \textit{IDH1R132}, \textit{IDH2R140} and \textit{IDH2R172} mutations as it showed 100% specificity, 100% sensitivity, and a LOD estimated at 0.5% and 1.2% on \textit{IDH1R132}, \textit{IDH2R140}, and \textit{IDH2R172} codons for blood/bone marrow samples and FFPE samples respectively. While the PGX PCR assay is not quantitative, \textit{IDH1/2} mutant frequencies measured by ddPCR were linear between 1% and 45.6% and showed a high correlation with VAF obtained by NGS results. In contrast, the sensitivity of
the automated multiplex PCR easy-PGX was lower, thus limiting its potential for IDH1/2 hotspot codon screening on clinical samples. Indeed, PGX failed to detect two mutations at codon 140, whose VAFs were 4.8% and 6%, while the supplier’s LOD was 2%. Similarly, PGX failed to detect 10/15 IDH2R172 mutations, including 5/8 IDH1R132 and IDH2R140 mutated samples tested by PGX were extracted from blood or bone marrow samples. PGX sensitivity was higher on these samples at 100% and 84.4% for marrow samples. PGX sensitivity was higher on these samples at 100% and 84.4% for IDH1R132 and IDH2R140, respectively (Table S1). Notably, most IDH2R172 mutated samples genotyped by PGX (n = 11/15) were extracted from T-cell lymphoma FFPE samples. Conversely, IDH1R132 and IDH2R140 mutated samples tested by PGX were extracted from blood or bone marrow samples. PGX sensitivity was higher on these samples at 100% and 84.4% for IDH1R132 and IDH2R140, respectively. These results suggest that PGX sensitivity may be significantly altered on FFPE samples. The PGX LOD was estimated using commercial DNA or recombinant plasmids that may not be a good surrogate of the patient’s DNA, especially those obtained from FFPE tissues. These data underline the importance, for PGX assay, of re-evaluating the LOD on patient samples for each of the mutations tested.

Finally, in hematological malignancies, the VAF can be low (<5%), especially for patients’ follow-up after anti-IDH therapy or in AITL patients [28]. In terms of sensitivity, ddPCR assay has proven to be the best method compared with PGX qPCR with a LOD estimated at 0.5% for blood/bone marrow samples and 1.2% for FFPE samples. However, it must be noted that the multiplexed ddPCR assay can detect a mutated codon but does not identify the exact mutation. In addition, this assay does not detect rare mutations such as IDH2R172T. Nevertheless, according to the COSMIC (Catalogue Of Somatic Mutations In Cancer) database, this design allows the detection of 99.8% and 98.9% of the mutations identified for the IDH1 and IDH2 genes, respectively (Table S3).

5. Conclusions

The multiplexed ddPCR assay evaluated is a fast and quantitative method that significantly spares technical time and reduces analysis cost compared with NGS. It also covers most IDH1/2 mutations with a LOD adapted to patient sample screening and therefore could meet most clinical needs of cancer molecular diagnostics laboratories.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

AP and IS supervised and designed the experiments for PGX qPCR and ddPCR, respectively. NS, ST, CJ, and VTQ performed the experiments. LF, NS, ST, AP, EG, OW-B, PG, AD and IS analyzed and interpreted data. LF, AP, and IS wrote the manuscript with OW-B, PG, NS, AD, and ST inputs. All authors approved the manuscript.

Data availability statement

The supporting data are available in the Table S1.

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**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Table S1.** Sample genotyping by NGS, PGX, and ddPCR.

**Table S2.** Mutations assessed by EasyPGX ready IDH1/2 kit.

**Table S3.** Mutations assessed by ddPCR.

**Table S4.** Diagnostic test values.

**Figure S1.** Detection of an additional mutation by ddPCR below the detection limit of NGS.