Novel method to detect microRNAs using chip-based QuantStudio 3D digital PCR

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Abstract

Background: Research efforts for the management of cancer, in particular for lung cancer, are directed to identify new strategies for its early detection. MicroRNAs (miRNAs) are a new promising class of circulating biomarkers for cancer detection, but lack of consensus on data normalization methods has affected the diagnostic potential of circulating miRNAs. There is a growing interest in techniques that allow an absolute quantification of miRNAs which could be useful for early diagnosis. Recently, digital PCR, mainly based on droplets generation, emerged as an affordable technology for precise and absolute quantification of nucleic acids.

Results: In this work, we described a new interesting approach for profiling circulating miRNAs in plasma samples using a chip-based platform, the QuantStudio 3D digital PCR. The proposed method was validated using synthetic oligonucleotide at serial dilutions in plasma samples of lung cancer patients and in lung tissues and cell lines.

Conclusion: Given its reproducibility and reliability, our approach could be potentially applied for the identification and quantification of miRNAs in other biological samples such as circulating exosomes or protein complexes. As chip-digital PCR becomes more established, it would be a robust tool for quantitative assessment of miRNA copy number for diagnosis of lung cancer and other diseases.

Keywords: miRNA, Absolute quantification, Lung cancer

Background

MicroRNAs (miRNAs) are small non-coding RNAs, 19–24 nt-long, tissue specific, that regulate gene expression by post-transcriptional regulation [1, 2]. They play a critical role in development and differentiation processes of tissues and organs and are aberrantly expressed in different kinds of cancer [3, 4]. Over the last few years several studies have shown that miRNAs can be detected within body fluids such as plasma [5], serum [6], sputum [7], saliva [8], and urine [9]. Although the mechanism of secretion and incorporation of miRNAs has not been fully clarified, circulating miRNAs may play a pivotal and general role as signaling molecules in physiological and pathological events [10].

Notably, circulating miRNAs levels were found to correlate with cancer progression, therapeutic response, and patient survival, suggesting that they could also be used as non-invasive biomarkers [11, 12].

Lung cancer is the leading cause of cancer deaths in the world due to its high incidence and mortality, with 5-year survival estimates around 15 % for non-small-cell lung cancer (NSCLC) [13]. Despite recent advances in the management of lung cancer and the use of molecular targeted agents in specific clinical settings, the cure rate remains low due to drug-refractory recurrent disease [13].

We previously identified in tumor, normal lung tissue and plasma samples miRNA signatures with diagnostic and prognostic potential [14]. In a recent study we showed that the combination of low dose computed tomography (LDCT) screening and our plasma miRNA signatures reduced LDCT false positives in a retrospective screening series of more than 1000 individuals [15].

The high potential of the circulating miRNAs as molecular marker of disease is diminished by a lack of consensus regarding an optimal method of normalization in
plasma samples. The small-nucleolar RNAs (snoRNAs), such as RNU6B and RNU48, cannot be used to this purpose due to the absence in plasma [16]; other housekeeping miRNAs candidate have been proposed in different studies but a global consensus on their use is still lacking [17, 18]. Alternatively, for assay with a relatively large number of miRNAs, a normalization using the mean or the median could be applied [19]. Our group described an approach for circulating miRNAs profiling in plasma samples based on the evaluation of 24 miRNAs reciprocal levels measured by quantitative Real-Time PCR [20]. Recently, it was suggested an absolute quantification of miRNAs using RT-PCR with a standard curve generated with a synthetic oligonucleotide but this approach could be useful only for individual miRNA quantification but not for multiplex miRNA evaluation. Furthermore, other groups proposed a normalization method based on the addition of a spike-in miRNAs. They recommended the use of C. elegans control miRNAs during the denaturation of samples to normalize the variability that could affect the reaction efficiency [21].

Digital PCR (dPCR) is an end-point PCR method that is used for absolute quantification. The dPCR concept was conceived in 1992 [22] and it was used to quantify KRAS mutations in DNA from colorectal cancer patients [23]. Digital PCR has many potential applications, including the detection and quantification of low-level pathogens [24], rare genetic sequences [25], copy number variations (CNVs) [26], gene expression in single cells [27] and quantification of circulating miRNAs expression [28, 29].

The following is a table of the PCR run protocol:

| Step type     | Time   | Temperature (°C) |
|---------------|--------|-----------------|
| Hold          | 10 min | 96              |
| Cycle (40 cycles) | 2 min | 56               |
|               | 30 s   | 98              |
| Hold          | 2 min  | 60              |
| Hold          | ∞      | 10              |

Fig. 1 Workflow of a digital PCR experiment

Table 1 PCR run protocol
In this work, we propose for the first time, a methodological workflow, shown in Fig. 1, for the absolute quantification of miRNAs, in plasma or tissue/cells of lung cancer patients using a chip-based platform, the QuantStudio 3D Digital PCR. For the description of the results we adopted the guidelines for the publication of digital PCR experiments described by Huggett et al. [30]. This innovative data analysis tool allowed us to circumvent the normalization issue and given the high reproducibility of this procedure, we believe that it could be routinely used for the analysis of miRNAs also in cancer clinical series.

**Methods**

**Samples**

**Plasma samples**

Plasma samples were collected from high-risk heavy smoker volunteers aged from 50 to 75 years, including current or former smokers with a minimum pack/year index of 30 enrolled in a LDCT screening trial (BioMild) performed at our Institution [31].

**Table 2** dPCR detection efficiency

| Dilution | Cell-mir-39 copies/ul expected | Cell-mir-39 copies/ul observed | Detection efficiency (%) |
|----------|--------------------------------|-------------------------------|--------------------------|
| D1       | 500                            | 598                           | 83                       |
| D2       | 100                            | 115                           | 87                       |
| D3       | 20                             | 25,24                         | 78                       |
| D4       | 4                              | 5,4                           | 74                       |
| D5       | 0,8                            | 1,08                          | 74                       |
| D6       | 0,15                           | 0,13                          | 115                      |

**Fig. 2** Quantification of synthetic oligonucleotides by dPCR. Analysis of five-fold dilution series of cel-mir-39 using FAM and VIC probes. Representative plot of dPCR (left panels) and number of copies/ul (right panel) were shown. The data points in the plot are color-coded according to the following call types: FAM (blue), VIC (red), FAM + VIC (green) and NOT AMPLIFIED (yellow).

**Fig. 3** Absolute quantification of miRNA expression levels. **a** Bar graphs show the number of copies/ul for mir-16-5p, mir-21-5p, mir-126-3p, mir-486-5p and mir-660-5p in three different samples (plasma, tissue and cells) \((n = 3)\). Data are expressed mean ± standard error of the mean. **b** mir-16-5p expression in different specimens: healthy peripheral blood mononuclear cells (PBMC), normal lung tissues and cell lines from breast cancer, fibroblast and osteosarcoma. The experiments were done in duplicates.
**Lung cancer cell lines**

Human lung cancer cell lines, A549 and H1299, were obtained from the American Type Culture Collection (ATCC). LT73 cells were derived in our laboratory from a primary lung tumor of a 68-year-old Caucasian male with lung adenocarcinoma [32].

**PDX**

Lung cancer patients' derived xenografts (PDXs) were developed by directly implanting fragments of the patient's living tumor in the flanks of immunocompromised mice [33].

**Ethics statement**

Tissue and plasma specimens were obtained according to the Internal Review and the Ethics Boards of the Istituto Nazionale Tumori of Milan (INT 2111). All patients provided informed consent.

**MicroRNA profiling**

**Taqman assays**

MiRNA expression was analyzed using Taqman MicroRNA assays (Thermo Fisher Scientific): mir-16-5p (ID:000391), mir-21-5p (ID:000397), mir-126-3p (ID:002228), mir-486-5p (ID:001278), mir-660-5p (ID:001515), cel-mir-39 (CUSTOM) and RNU48 (ID:001006).

**Plasma**

Total RNA was extracted from 200 μl plasma samples using mirVana PARIS Kit (Thermo Fisher Scientific), according to the protocol for biological fluids. Synthetic *C. elegans* miRNA-39 (cel-miR-39) was used as spiked-in control, adding to each plasma sample 5 μl from a 5 fmol/μl stock tube (Qiagen). Samples were eluted in 50 μL of Elution Solution pre-heated at 95 °C to obtain more concentrated total RNA.

RT reaction was performed on 3 μl of total RNA, using the TaqMan MicroRNA Reverse Transcription Kit.

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![Fig. 4](image.png)

**Fig. 4** miRNAs expression levels in plasma samples. Bar graphs (left) show number of copies/μl of each miRNAs analyzed of three different plasma samples and their respective ten-fold dilution. Representative dPCR plots of the miRNA analysis (right). The data points in the plot are color-coded according to the following call types: FAM (blue), VIC (red), FAM + VIC (green) and NOT AMPLIFIED (yellow).
and a Custom TaqMan RT Primer Pool (Thermo Fisher Scientific), according to the manufacturer’s instruction. Since we started from a small amount of total RNA, a pre-amplification step of 12 cycle was required, thus 2.5 μL of each RT product were pre-amplified using a Custom TaqMan PreAmp primer pool (Thermo Fisher Scientific).

Tissues and cultured cells
PDX tissue samples were disrupted and homogenized using 3 mm Tungsten Carbide Beads and the Mixer Mill MM300 (Qiagen). For tissue samples and cultured cells, total RNA was extracted using mirVana PARIS Kit (Thermo Fisher Scientific) following manufacturer’s instructions. Cel-miR-39 was used as spiked-in control, by adding to each samples 5 μl from a 5 fmol/μl stock tube. Total RNA was quantified with the NanoDrop 2000 (Thermo Fisher Scientific).

Starting from 100 ng of total RNA, reverse transcription was performed using the TaqMan microRNA Reverse Transcription Kit and a TaqMan RT Primer Pool with the miRNAs of interest according to the manufacturer’s instruction (Thermo Fisher Scientific).

PCR on chip
We combined 2,25 μl of RT product (RT or PreAmp product obtained in the previous step) with 3,75 μl nuclease-free H2O, 7,50 μl QuantStudio™ 3D Digital PCR Master Mix, 0.75 μl of TaqMan MicroRNA Assay-1 (20X) and 0.75 μl TaqMan MicroRNA Assay-2 20X (cel-miR-39-VIC).

To avoid pipetting errors, we prepared a stock solution and we included 10 % excess for volume loss from

| Table 4 | Pearson correlation in plasma samples between assays and samples |
|---|---|---|---|---|---|---|---|
| Real Time PCR (Ct) | miR-16 | miR-126 | miR-486 | miR-21 | miR-660 | Pearson correlation |
| PLASMA 1 | 17,86 | 19,87 | 20,83 | 24,63 | 28,57 | –92 % |
| PLASMA 2 | 18,39 | 19,58 | 20,38 | 24,32 | 28,12 | –89 % |
| PLASMA 3 | 18,59 | 18,88 | 21,35 | 24,66 | 28,76 | –83 % |
| dPCR (copies/ul) | miR-16 | miR-126 | miR-486 | mir-21 | mir-660 | Pearson correlation |
| PLASMA 1 | 7590 | 4746,84 | 2882 | 755,79 | 37 | –99 % |
| PLASMA 2 | 6766 | 3261,78 | 3201 | 1855,87 | 41 | –76 % |
| PLASMA 3 | 6230 | 6840,79 | 643 | 913,46 | 27 | –93 % |
| Pearson correlation | –99 % | –76 % | –93 % | –98 % | –87 % |
pipetting. This sample mix was added on each chip and loaded on ProFlex™ 2x Flat PCR System with the following program (Table 1):

Absolute quantification was determined using QuantStudio 3D Digital PCR System (Thermo Fisher Scientific) and analyzed with QuantStudio 3D AnalysisSuite Cloud Software (Thermo Fisher Scientific).

**Results and discussion**

**Chip quality control**

The software assesses whether the data on a chip is reliable based upon loading, signal, and noise characteristics and displays quality indicators for each chip in a project.

This quality control is based on the number of partitions that exceed the selected quality threshold (fixed automatically at 0.5) on the total number of wells filled correctly. To get a precise quantification we settled a threshold of 10,000 data points for quality control of the chip (Additional file 1: Figure S1).

**Use of spike-in (VIC fluorescence)**

Several studies report on the use of synthetic RNA or miRNA molecules as spike-in controls for mRNA/miRNA expression data normalization [34–36].

In the proposed method, we decided to add in the mix an exogenous spike-in miRNA (cel-mir-39 conjugated with VIC fluorescence) as internal control for efficiency of the whole reaction (from extraction to PCR) since the fluorescence analysis using only FAM probes did not allow a precise quantification of miRNAs.

To test the overall performance of the method in terms of efficiency, precision, and sensitivity we generated a standard curve using a serial dilution of cel-mir-39 mimic.

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![Graphs](image)

**Fig. 5** Absolute quantification of miRNAs in lung cancer cells. Bar graphs (left) show number of copies/μl of mir-16, mir-486 and mir-660 in H1299 (upper), A549 (middle) and LT73 (lower) cell lines and their respective ten-fold dilution. Representative dPCR plots of the miRNA analysis (right). The data points in the plot are color-coded according to the following call types: FAM (blue), VIC (red), FAM + VIC (green) and NOT AMPLIFIED (yellow)
We generated a curve consisting in six points of a five-fold serial dilution, starting from 5 fmol of cel-mir-39 mimic. In this way the copy number of each single miRNAs measured displayed a good linear response to input mimic amount (Fig. 2). Furthermore, we calculated the expected copies for each dilution of cel-mir-39 as described by Hindson et al. [37] to show dPCR detection efficiency assuming 100 % RT efficiency and an accurate known concentration of the synthetic miRNA supplied by the vendor (Table 2). We noted that absolute measurements by dPCR corresponded to 74–115 % of the theoretically input copies, indicating that absolute detection by dPCR is remarkably efficient.

miRNA expression analysis

We decided to use our methodology for the quantification of miRNAs levels in different type of samples (plasma, tissue and cells) and its potential utility for cancer diagnosis of our 24 miRNAs composing the diagnostic signature [15]. As shown in Fig. 3a, we analyzed the expression of five miRNAs (mir-16-5p, mir-21-5p, mir-126-3p, mir-486-5p and mir-660-5p) already demonstrated to have a role in lung cancer development [32, 38–41]. This proposed method could be useful for the quantification of miRNA levels in various samples from normal or pathological conditions. To demonstrate the potential use of this protocol, we analyzed the expression of mir-16-5p, an ubiquitous miRNA, in different specimens as healthy peripheral blood mononuclear cells (PBMC), normal lung tissue and cell lines of breast cancer, fibroblast and osteosarcoma (Fig. 3b).

Plasma analysis

To evaluate the sensitivity and the precision of our digital PCR methods, we select three of the selected miRNAs showing different levels of expression (i.e. mir-16-5p mir-486-5p and mir-660-5p) and analyzed their expression in plasma samples. On the basis of the expected circulating miR-16-5p levels, we performed a five-fold dilution of the samples to have high efficiency of quantification compared to the undiluted sample (data not shown for undiluted sample). For the other two miRNAs, mir-486-5p and mir-660-5p, no dilution was required (Fig. 4 and Table 3). To compare results obtained with qPCR, we tried to correlate raw Ct data for these five circulating miRNA obtained with custom microfluidic cards [20] and the number of copies obtained with dPCR (Table 4) assuming 100 % primers efficiency as described by the vendor. As reported in Table 4, we observed a good correlation intra-run assay and between samples for circulating miRNAs analyzed.

To demonstrate the specificity of the absolute quantification, we performed a ten-fold dilution for each samples and we confirmed that there was a linearity for each miRNAs between the two samples (fold-dilution average between samples: mir-16-5p: 8,81 ± 0,675, mir-486-5p: 7,67 ± 2,41, mir-660-5p: 9,96 ± 1,76).

### Table 5

| Sample | Assay | cel-mir-39 | CI- cel-mir-39 | Target miRNA | CI- miRNA | Data points above threshold (0.5) |
|--------|-------|------------|----------------|--------------|-----------|----------------------------------|
| H1299  | 16–39 | 981,8      | 962,5 – 1001,5 | 5025,6       | 4880,6 – 5175 | 18,079 of 18,671                |
| H1299  | 486-39| 940,9      | 921,7 – 960,6  | 17,1         | 15,1 – 19,4  | 17,084 of 19,738                |
| H1299  | 660-39| 1,275,7    | 1,252 – 1,299,8| 112,7        | 107,5 – 118,2| 18,148 of 19,385                |
| H1299 1:10 | 16–39 | 302,9      | 293,4 – 312,8  | 439,3        | 427,4 – 451,5 | 16,281 of 18,954                |
| H1299 1:10 | 486-39| 345,8      | 335,8 – 356,1  | 1,8          | 1,2 – 2,6     | 17,328 of 18,723                |
| H1299 1:10 | 660-39| 334,3      | 324,4 – 344,5  | 9,2          | 7,7 – 10,9    | 16,976 of 18,006                |
| A549   | 16–39 | 808,9      | 790,1 – 828,3  | 2130,9       | 2087,6 – 2175,1| 14,270 of 19,057                |
| A549   | 486-39| 1309,6     | 1285,3 – 1334,3| 19,9         | 17,8 – 22,3   | 18,008 of 19,310                |
| A549   | 660-39| 1,403,2    | 1,372,5 – 1,434,6| 69          | 64,0 – 74,0   | 12,610 of 17,988                |
| A549 1:10 | 16–39 | 486,6      | 473,6 – 499,9  | 483,4        | 470,5 – 496,7 | 15,486 of 16,723                |
| A549 1:10 | 486-39| 401,3      | 379,9 – 423,8  | 1,57         | 0,7 – 3,5     | 17,285 of 18,655                |
| A549 1:10 | 660-39| 367,4      | 355,9 – 379,5  | 5,36         | 4,1 – 6,8     | 13,841 of 17,322                |
| LT73   | 16–39 | 655,2      | 640,7 – 670,1  | 3266,7       | 3200,2 – 3334,5| 18,070 of 19,251                |
| LT73   | 486-39| 921,1      | 902,2 – 940,4  | 16,5         | 14,6 – 18,8   | 17,170 of 18,120                |
| LT73   | 660-39| 574,0      | 558,7 – 589,8  | 4,0          | 3,0 – 5,3     | 13,606 of 19,582                |
| LT73 1:10 | 16–39 | 90,4       | 85,5 – 95,5    | 409,9        | 398,6 – 421,5 | 16,673 of 18,990                |
| LT73 1:10 | 486-39| 66,8       | 62,7 – 71,1    | 1,3          | 0,9 – 2,1     | 17,359 of 18,326                |
| LT73 1:10 | 660-39| 60,8       | 57,0 – 64,8    | 0,3          | 0,1 – 0,8     | 18,164 of 19,404                |
The number of copies per μl between samples was normalized based on the mean of cel-mir-39 expression for each chip and multiplied by the number of dilution folds in the plasma.

We replicated miRNAs expression analysis on three different plasma samples and we obtained similar data of expression for each miRNAs (Fig. 4 and Table 3). Negative and non-template controls for each miRNA were run on chip and did not show any positive results.

**Cell analysis**

To demonstrate the potential use of QuantStudio 3D digital PCR for cellular miRNAs expression analysis, we analyzed the levels of these miRNAs in three different lung cancer lines. As already described, mir-16-5p is one of the most abundant miRNA in lung cancer cells, whereas mir-660 and mir-486-5p expression is very low. Starting from 100 ng of total RNA, we quantified the miRNA expression levels using undiluted or ten-fold diluted samples (Fig. 5 and Table 5). As shown in the graphs the sensitivity of the method was confirmed also for miRNA cellular expression (fold-dilution average between samples: mir-16-5p: 10.45 ± 3.87, mir-486-5p: 12.45 ± 2.95, mir-660-5p: 10.99 ± 3.69). Moreover, we performed RNU48 analysis cell samples and we observed that the number of copies for RNU48 was similar for all the samples starting from the same amount of input RNA (data not shown). Negative and non-template controls for each miRNA were run on chip and did not show any positive results.

**PDX analysis**

Our method could be applied for the analysis of miRNAs expression in tissues and to demonstrate this potential use we performed the same analysis described above. We extracted RNA and measured miRNAs levels from three different samples of our patients derived xenografts. As described in Fig. 6 and Table 6, mir-16

![Figure 6](image-url)  
**Fig. 6** Number of miRNAs copies per μl in lung PDX tissues. Bar graphs (left) show the expression levels of the miRNAs analyzed in three different samples of PDX and their respective ten-fold dilution. Representative dPCR plots of the miRNA analysis (right). The data points in the plot are color-coded according to the following call types: FAM (blue), VIC (red), FAM + VIC (green) and NOT AMPLIFIED (yellow).
was the most expressed miRNAs in tissues whereas miR-486 and miR-660 had similar expression levels. Ten fold dilution of the samples confirmed the sensitivity of the analysis in tissues (fold-dilution average between samples: mir-16-5p: 9,92 ± 2,18, mir-486-5p: 10,39 ± 1,32, mir-660:-5p 11,03 ± 1,92). As described above, we performed RNU48 analysis on all tissues and we observed that the number of copies for RNU48 was similar for all the samples starting from the same amount of input RNA (data not shown). Negative and non-template controls for each miRNA were run on chip and did not show any positive results.

**Precision of the methodology**

To determine repeatability of the methods we performed mir-16-5p analysis on 5 different samples in duplicates and measured the coefficient of variation [37] in the same PCR run. The same samples were also analyzed in two different PCR runs to determine the overall precision of the methodology. As shown in Table 7, digital PCR results displayed low variation both intra and inter-run replicates (% CV within-run: 4 %; overall precision: 4 %) (Table 7).

**Conclusion**

In this work, we developed for the first time a new method for the detection of miRNAs in different type of biological specimen as plasma, cell lysates or tissue. This method could be particularly useful for quantification of miRNAs in those biological samples, such as plasma/serum or exosomes, where lack of consensus for the normalization strategy prevents clinical applications.

Digital PCR has a great potential but several tips need to be followed: first a rough estimate of the concentration of your target of interest has to be previously done in order to make appropriate dilutions. Otherwise, it is possible that too many partition will get multiple copies preventing an accurate calculation of the copy number of your miRNA. Furthermore, non-template controls and a RT negative control must be set up for each primers pool methods for retro-transcription.

A chip-based digital PCR approach has the advantage to require less pipetting steps and to reduce PCR contamination. In comparison, the droplet digital PCR requires multiple pipette transfers that could potentially increase the risk of contamination. Furthermore, QuantStudio 3D chips have 20.000 fixed reaction wells whereas Table 6 miRNAs levels in PDXs tissues

| Sample | Assay | cel-mir-39 | CI- cel-mir-39 | Target miRNA | CI- miRNA | Data points above threshold (0.5) |
|--------|-------|------------|----------------|--------------|----------|----------------------------------|
| PDX 1  | 16–39 | 430,6      | 418 – 443,5    | 968,4        | 946,9 – 990,4 | 14,289 of 18,775 |
| PDX 1  | 486-39| 682,8      | 666,8 – 699,1  | 45,2         | 41,8 – 48,9  | 15,843 of 18,068  |
| PDX 1  | 660-39| 521,2      | 507,9 – 534,9  | 18,8         | 16,7 – 21,2  | 16,106 of 18,346  |
| PDX 1:10| 16–39 | 122,4      | 105,8 – 141,7  | 162,29       | 155,7 – 169,2| 18,068 of 19,395  |
| PDX 1:10| 486-39| 58,0       | 54,3 – 62,1    | 2,99         | 2,2 – 3,9    | 17,381 of 19,135  |
| PDX 1:10| 660-39| 52,7       | 47,5 – 58,4    | 1,72         | 0,9 – 3,0    | 17,868 of 18,826  |
| PDX 2  | 16–39 | 601,9      | 586,6 – 617,7  | 937,7        | 916,8 – 958,9| 14,472 of 16,749  |
| PDX 2  | 486-39| 532,9      | 520,1 – 546,1  | 23,8         | 21,5 – 26,4  | 17,831 of 19,264  |
| PDX 2  | 660-39| 441,6      | 426,6 – 457,2  | 23,2         | 20,2 – 26,5  | 10,233 of 15,163  |
| PDX 2:10| 16–39 | 59,7       | 55,9 – 63,8    | 134,7        | 128,8 – 140,9| 17,341 of 19,135  |
| PDX 2:10| 486-39| 57,5       | 53,2 – 62,2    | 1,5          | 0,9 – 2,4    | 13,040 of 18,311  |
| PDX 2:10| 660-39| 62,3       | 58,3 – 66,4    | 2,8          | 2,1 – 3,8    | 17,358 of 19,226  |
| PDX 3  | 16–39 | 83,5       | 78,9 – 88,4    | 798,1        | 781,0 – 815,5| 17,174 of 19,355  |
| PDX 3  | 486-39| 104,7      | 98,6 – 111,2   | 9,9          | 8,2 – 12,0   | 12,183 of 18,035  |
| PDX 3  | 660-39| 86,1       | 81,5 – 91,1    | 9,0          | 7,6 – 10,7   | 17,004 of 18,146  |
| PDX 3:10| 16–39 | 12,9       | 11,2 – 14,8    | 90,5         | 85,6 – 95,5  | 17,089 of 18,817  |
| PDX 3:10| 486-39| 7,3        | 6,7 – 8,7      | 0,5          | 0,3 – 1,0    | 17,966 of 19,844  |
| PDX 3:10| 660-39| 7,7        | 6,4 – 9,2      | 0,5          | 0,3– 1,1     | 17,509 of 19,282  |

**Table 7 Coefficient of variation between dPCR replicates**

| Sample  | CV across intra-run replicates | CV across inter-run replicates |
|---------|--------------------------------|--------------------------------|
| Sample 1| 0,004                          | 0,05                          |
| Sample 2| 0,033                          | 0,02                          |
| Sample 3| 0,009                          | 0,02                          |
| Sample 4| 0,008                          | 0,07                          |
| Sample 5| 0,142                          | 0,04                          |
| Average | 0,039                          | 0,040                         |
droplet PCR rely upon the generation of droplets, a step that could be extremely variable. Overall, our proposed method for miRNA quantification using a chip-dPCR were comparable in terms of accuracy and precision to the study reported by Miotto et al. using droplet digital PCR. (% CV within-run: chip 4 % droplet 5.1; overall precision: chip 4 % and droplet 13 %) [42].

Obviously, this approach has some limitations, for example, the ability to perform only one sample per chip, although it is possible to load in the thermocycler up to 24 chips. Moreover, using this approach tests in multiplex fluorescence can be carried out but only with two probes per chip. To date, dPCR could be potential useful for clinical diagnostic purpose only for small scale samples but we believe that, during the next years, the improvement of the methodology could permit multiplexing analysis. At the time, the instrument is not able to perform an accurate analysis when there is only one fluorescence, VIC or FAM, because it is set to choose a threshold for both fluorescence. In our methods, to solve this technical problem, we decided to put a reference control, an exogenous spike-in with VIC probe, which allows an accurate miRNA copy number quantification and also to have control of the whole process. A new version of the analysis software, now released, permit manual modifications of the fluorescence parameters such as threshold or scale of the axis. Nonetheless, the instruments take up to three minutes for the reading and the analysis of one chips and thus can be potentially used in a clinical diagnostic setting.

**Additional file**

**Additional file 1: Figure S1.** Review quality of dPCR chip. Representative images shown chips with good quality as data points above threshold or by calls (upper) and chips below the sufficient quality for miRNAs detection (lower). The data points in the plot are color-coded according to the following call types: FAM (blue), VIC (red), FAM + VIC (green) and NOT AMPLIFIED (yellow). (BMP 1250 kb)

**Competing interests**

The authors declare no conflict of interest.

**Authors’ contributions**

DC, CV, UP, GS and OF contributed to conception and design of this research and to interpretation of results. DC, CV, CB, PS and OF performed the experiments and analyzed the data. DC, UP, OF, and GS drafted the manuscript. All authors participated in the critical revision of the report. All authors read and approved the final manuscript.

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