Accurate Quantitation of miRNA by chip-based digital PCR

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs that function to regulate gene expression at transcriptional and post- transcriptional level. Mature miRNAs are short, usually 18-23 nucleotides in length, with high homology in the seed region (position 2-8 from the 5' end of a mature miRNA) within each family. This presents a great challenge when designing assays for quantitation by real-time polymerase chain reaction (PCR). Existing miRNA assays often trade in sensitivity and/or PCR efficiency for specificity, which leads to variation in assay characteristics. Comparison between two different mature miRNAs with quantitative real-time PCR (qPCR) requires generating standard curves and characterizing PCR efficiency and sensitivity. Here we report a solution based on QuantStudio™ 3D platform, a chip-based digital PCR technology. With this technology, we are able to demonstrate accurate quantitation of miRNAs without characterizing and optimizing the assay.

Briefly, we mixed the same amount (ratio=1:1) of synthetic miRNA of hsa-miR-19b and hsa-miR-92a. Following reverse transcription, we quantitated the miRNAs using qPCR and chip-based QuantStudio™ 3D digital PCR (dPCR). Data from qPCR showed a delta Ct at 1.84 ± 0.08 between the targets. In linear scale, this converted to a 3.59 fold difference between the two mature miRNAs, which deviated 259% from the input ratio, which equals 1. When examined with dPCR, the data indicated that the ratio between the amounts of the two mature miRNAs was 0.94 ± 0.02. Although statistically different from the input ratio, the results from dPCR deviated only 6% from the input ratio. Compared to qPCR, dPCR significantly increased the precision and accuracy in sample quantitation.

In summary, our study demonstrated that QuantStudio™ 3D platform, a chip-based dPCR technology, can greatly increase accuracy in quantitating miRNA without a complicated workflow. The simplified workflow and improved results of dPCR will be very useful features in broad applications such as revealing small changes in the miRNA expression levels.

INTRODUCTION

qPCR is widely used for quantitate DNA target molecules. Its major advantage is the large dynamic range and great linearity. The main application is for relative quantitataion with $\Delta\Delta C_t$ method. The relationship between sample input amount and C_t is affected by many factors, such as PCR efficiency. When it comes to absolute quantitation, standard curve method with known calibration standard is necessary to correct for those factors. This demands a complicated workflow and maintenance of standards.

Digital PCR is a paradigm in quantitative method. The quantitative precision comes from Poison Distribution for rare events and no long relies on PCR efficiency for quantitation precision. This is a main differentiating factor between dPCR and qPCR. The quantitation is only the presence or absence of target molecules.

Here we demonstrate the differences between dPCR and qPCR in quantitation of miRNA target. dPCR demonstrated higher precision and better accuracy than traditional qPCR.

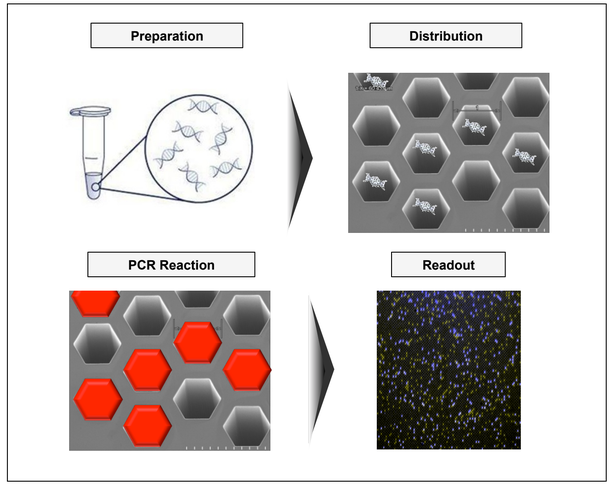
MATERIALS AND METHODS

Synthetic miRNA were ordered from Integrated DNA Technologies. Equal molar and a graduated amount of hsa-miR19b was mixed together with hsa-miR-92a to form test sample. miRNA assays were purchased from Life Technologies. Reverse transcription was done according to manufacture's protocol as custom RT pools[1].

qPCR was performed on ViiA7™ Real Time PCR system (Life Technologies, a Thermo Fisher Scientific Brand). Digital PCR was performed with QuantStudio™ 3D digital PCR system, according to manufacture's protocol[2]. Briefly, cDNA was diluted with 1x TE, pH 8.0 to a final dilution of 1/100,000. Diluted cDNA was mixed with miRNA TaqMan™ assay (final 1x) and QuantStudio™ 3D mastermix (final 1x) before loaded onto QuantStudio™ Digital PCR 20k Chip. For digital PCR, thermal cycle was performed on GeneAmp® PCR System 9700 for 40 cycles. Digital PCR 20k chip was then read on QuantStudio™ 3D digital PCR instrument.

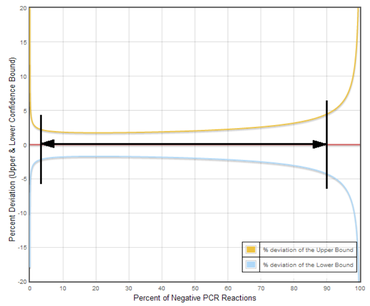
RESULTS

Figure 1. How Digital PCR Works



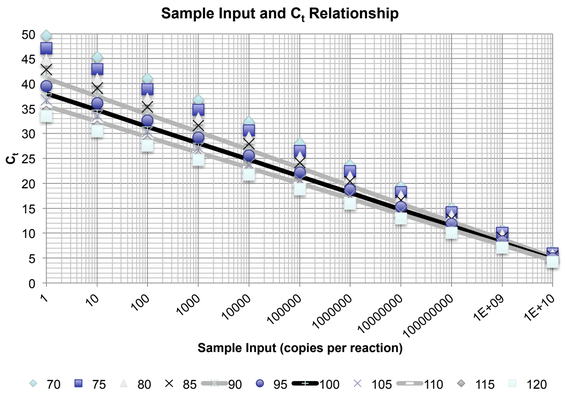
QuantStudio™ 3D is a chip-based digital PCR platform. The tightly packed hexagon-shaped well enables 20,000 individual reactions within in 1 mm² area. When reaction is loaded onto Digital PCR 20k Chip, the target molecules will be loaded into the wells following Poisson distribution. After amplification and TaqMan® probe hydrolysis, total number of reaction and negative wells will be used to calculate the mean target molecule concentration.

Figure 2. Digital PCR quantitation precision.



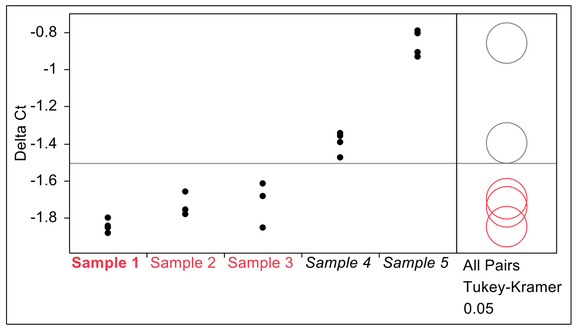
Digital PCR quantitation and relationship with percent of negative PCR reactions. The confidence interval at 95%, expressed at percent deviation, is affected by total number of reaction and percent of negative PCR reactions. As indicated in the chart, the percentage deviation is asymmetrically centered around 20% negative PCR reaction.

Figure 3. Sample Input and Ct of qPCR



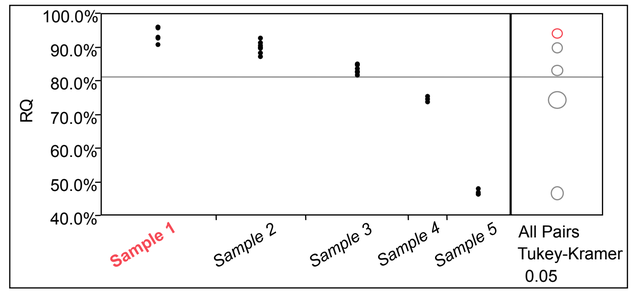
In quantitative PCR reaction, C_t is a function of sample input and PCR assay efficiency. For a typical PCR assay with efficiency at 100% (black line), "single" copy of target molecule will have Ct around 38. Various levels of sample input were simulated with different PCR efficiencies. Legend: PCR efficiency (%). The two grey lines indicates assays with PCR efficiency at 90% and 110%.

Figure 4. qPCR quantitation of miRNA target hsa-miR-19b and hsa-miR-92a



Quantitation precision comparison between digital PCR and real-time qPCR. Sample 1 through 5 is a mixture of synthetic miRNA of hsa-miR-19b and hsa-miR-92 at different ratio: sample 1, 100%; sample 2, 95%; sample 3, 90%; sample 4, 75%; sample 5, 50%. After reverse transcription, cDNA was run with qPCR and digital PCR with QuantStudio 3D system. Delta Ct of qPCR between hsa-miR-19b and hsa-miR-92 were reported for each sample. Real-time qPCR was not able to discriminate a 10% difference, such as between sample 1 and sample 3, and below. Tukey-Kramer HSD test was done within JMP software with experiment replicates.

Figure 5. dPCR quantitation of miRNA target hsa-miR-19b and hsa-miR-92a



Quantitation precision comparison between digital PCR and real-time qPCR. Sample 1 through 5 is a mixture of synthetic miRNA of hsa-miR-19b and hsa-miR-92 at different ration: sample 1, 100%; sample 2, 95%; sample 3, 90%; sample 4, 75%; sample 5, 50%. After reverse transcription, cDNA was run with qPCR and digital PCR with QuantStudio 3D system. The relative quantitation results of digital PCR were reported in percentile for each sample. Digital PCR with QuantStudio 3D is able to discriminate a 5% difference between sample 1 and 2 (indicated by non-overlapping circles by Tukey-Kramer HSD test). Tukey-Kramer HSD test was done within JMP software with experiment replicates.

CONCLUSIONS

QuantStudio™ 3D, a chip-based digital PCR platform, provides a simpler workflow and better quantitation precision than traditional qPCR, such as 5% difference.

dPCR is less sensitive to variation of PCR efficiency than qPCR for relative quantitation.

dPCR provides accurate quantitation of miRNA, reflecting the input amount of RNA.

dPCR is capable to discriminate sample with smaller differences with high precision than qPCR.

REFERENCES

1. Protocol for Creating Custom RT and Preamplification Pools using TaqMan MicroRNA assays. http://tools.lifetechnologies.com/content/sfs/manuals/cms_094060.pdf

2. User manual for QuantStudio™ 3D digital PCR system. http://tools.lifetechnologies.com/content/sfs/manuals/MAN0007720.pdf

3. Portal page for QuantStudio™ 3D digital PCR system. http://www.lifetechnologies.com/quantstudio3d

4. Other Life Technologies AACR poster can be found: http://www.lifetechnologies.com/aacrposters

Note

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