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Comparison of Quantitative PCR and Droplet Digital PCR for Effective Hepatitis B Covalently Closed Circular DNA Measurement

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ABSTRACT

Chronic hepatitis B virus (HBV) infection is caused by the persistence of viral covalently closed circular DNA (cccDNA) in infected hepatocytes. Targeting cccDNA is a novel approach for chronic HBV infection treatments. Since the number of cccDNA presented in a hepatocyte is very low, the precise and sensitive technique has to be developed in order to detect and quantify cccDNA. Even though quantitative polymerase chain reaction (PCR) was used for almost 3 decades by fluorescent dyes or probes to detect the amplification of DNA target, this technique only provides a relative quantification that is dependent with calibration curve. This limitation was resolved by developing of droplet digital PCR (ddPCR). ddPCR offers an absolute quantification that calculated based on Poisson algorithm. Furthermore, it is advantageous by high sensitivity and reproducibility to detect low amplicon template with extreme precision. This study presents the successful validation of ddPCR for applying to estimate cccDNA copy number. By using known concentration of plasmid, ddPCR showed the lowest detection limit around 0.1 copies per 1X reaction volume. The accuracy to quantify cccDNA target was compared between theoretical and practical value and determined the correlation by R-squared close to 1. From our results, we exhibited that ddPCR should be a platform for quantifying cccDNA and applied in subsequent researches in order to evaluate the precise cccDNA number after cccDNA-targeting treatments.

Keywords: Covalently closed circular DNA, Quantitative PCR, Droplet digital PCR

INTRODUCTION

Chronic HBV infection leads to liver cirrhosis and hepatocellular carcinoma (HCC), the major causes of death in HBV infected patients (Organization, 2017). It remains one of the most serious liver infections worldwide because the available treatments can not completely eradicate the virus (Dawood et al., 2017; Yang et al., 2010). The key mechanism of chronic HBV infection (CHB) is the persistence of the viral DNA called cccDNA in the infected hepatocytes (Lucifora and Protzer., 2016) which promotes viral replication, new virion production, and subsequent HBV re-infection (Glebe and Bremer., 2013). Therefore, targeting cccDNA is a novel strategy for anti-CHB therapy. However, cccDNA is presented in HBV infected cell at very low copy number (Werle–Lapostolle et al., 2004). To achieve the strategy, the precise cccDNA measurement has to be developed. There are many methods that are used for DNA detection and quantification. Particularly, quantitative PCR (qPCR) is widely used by scientists, researchers, and clinicians for researches and clinical diagnostics. qPCR was firstly developed by adding ethidium bromide (EtBr), a fluorescent tag in molecular biology, to a PCR reaction. After amplification cycles, the fluorescent signal that could be detected during thermal cycling process was increased (Higuchi et al., 1992). Due to EtBr carcinogenicity, other safe fluorescent dyes, such as SYBR Green, EvaGreen, and SYTO9 (Monis, Giglio, and Saint, 2005), were developed and widely used as dye-based qPCR. Moreover, the specificity of qPCR could be raised by probe-based qPCR that prevented the non-specific PCR products contamination detection (Heid et al., 1996). Recently, the novel technique called ddPCR was developed. This technique provides high-precision of absolute quantification of DNA targets (Hindson et al., 2011), which is beneficial over qPCR which provides relative quantification. ddPCR is performed by partitioning PCR mixture into almost 20,000 oil droplets. Each droplet is independently amplified and detected the positive and negative fluorescent signals to indicate the availability of interested DNA target. Poisson distribution is used to analyzed the target copies in a microliter of the final 1X ddPCR reaction mix. Beside the absolute quantification with extreme accuracy, ddPCR is advantageous by high sensitivity and reproducibility to detect low amplicon template without the necessary of an external reference (Hindson et al, 2011; Strain et al, 2013; Gerdes et al, 2016; Pinheiro et al; 2011). In this study, qPCR assay were compared to ddPCR in order to quantify cccDNA from HepG2.2.15 cells, the HBV-producing cell line which contains cccDNA, in

further studies. The sensitivity and accuracy of both methods were evaluated using the known concentration of plasmid containing cccDNA sequences.

MATERIAL AND METHODS

PCR template preparation

HBV 1.3 mer WT replicon plasmid (Plasmid #65459) purchased from addgene was expanded by DH5α transformation, single colony culture, and plasmid isolation by Geneaid™ Plasmid Maxi Kit (Taiwan). The plasmid concentration was measured by NanoDrop 2000 Spectrophotometer, and qPCR templates were prepared by calculating the plasmid concentration to 10^{11} copies/µL as the initial concentration. The conversion formula is:

$$1 \text{ copy plasmid} = (1/6.02 \times 10^{23}) \times 6{,}821 \times 654.0 = 7.4 \times 10^{-6} \text{ pg plasmid}$$

While; 6.02×10^{23}, 6,821, and 654.0 are the Avogadro's constant, HBV 1. 3 mer length, and an average molecular weight of a pair of deoxyribonucleotide monophosphates, respectively.

There are 2 amplification sites of cccDNA primers in HBV 1.3 mer plasmid as shown in Figure 1. Therefore, the amplification value obtained from both qPCR and ddPCR will presented as a double copy of cccDNA. To achieve accurate results, the calculated copy number of plasmid used for PCR has to be multiplied by 2. For example, adding 7.4×10^{-6} pg plasmid DNA template will be equal to 2 copies of cccDNA.

The 2×10^{11} copies/ µL cccDNA template calculated from the molecular weight of plasmid was ten-fold serially diluted to 2×10^{-1} copies/µL and used as qPCR templates. To prepare ddPCR templates, the DNA had to be in a short, linear form for effective droplet generation. BglII restriction enzyme was chosen to cut HBV 1.3 mer plasmid because its restriction sites are outside the PCR targets. The plasmid DNA was calculated to the final concentration of 2×10^{11} copies/ µL in FastDigest BglII reaction. After completion of the restriction enzyme digestion step, cut DNA was ten-fold serially diluted to 2×10^{-1} copies/ µL as prepared for qPCR templates.

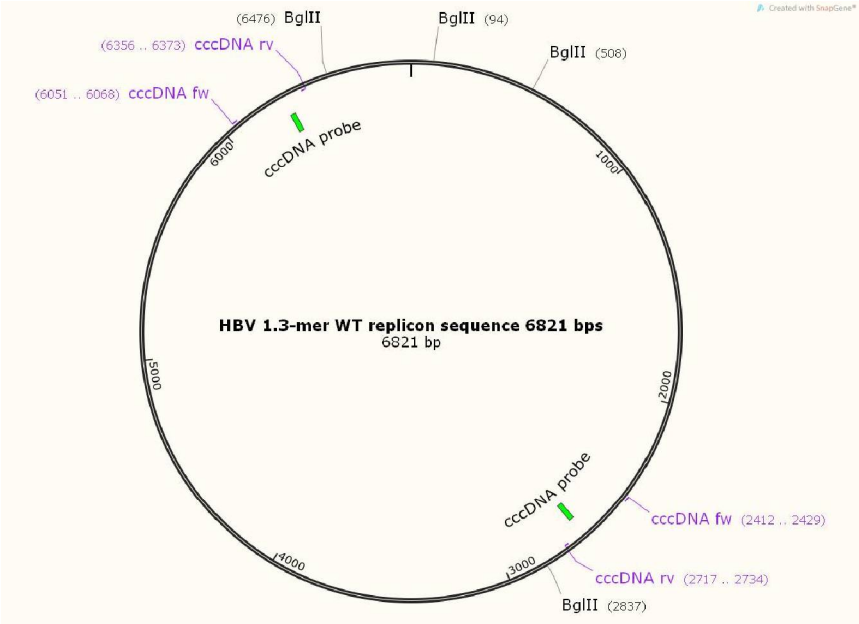

Figure 1. Circular map of HBV 1.3 mer WT replicon. cccDNA primers binding sites are shown at labels of cccDNA fw and rv. Green bars represent the binding sites of cccDNA probes which are correctly located at cccDNA targets. The digestion sites of BglII are also shown outside of the PCR targets.

Extracted cccDNA was used as the template in qPCR. While in ddPCR, BglII-cut cccDNA was used. Both templates were prepared in the PCR reaction with the same initial concentration.

Quantitative real-time PCR

The real-time PCR reaction was prepared following the manufacturer's instructions using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). Plasmid HBV 1.3 mer or extracted cccDNA were used as templates. cccDNA FW: 5'-CTTCTCATCTGCCGGACC-3' and cccDNA RV: 5'-CACAGCTTGGAGGCTTGA-3' were used as primers. To amplify the signal of increasing PCR products, cccDNA probe: FAM- 5'-AGGCTGTAGGCATAAATTGGTCT- 3'-BHQ was used. The thermal cycling condition was set as follows:

1) 50.0 ºC 2:00 minutes
2) 95.0 ºC 10:00 minutes
3) 95.0 ºC 0:15 minutes

4) 58.3 ºC 1:00 minute + plate read
5) Go to 3) for 39 more times
7) 4.0 ºC Forever End

Quantitative real-time PCR results were analyzed using Bio-Rad CFX Manager 3.1 software (Bio-Rad, USA). Moreover, PCR products were run on 1.5% (w/v) agarose gels by electrophoresis.

Droplet Digital PCR

The ddPCR mixture (20 µL) contained 900 nM of forward and reverse primers, 250 nM of probe, 1X ddPCR Supermix for Probes, and BglII-cut sample DNA. The mixture was then transferred to a DG8™ Cartridges for QX100™ (Bio-Rad, USA) and covered with a Droplet Generator DG8™ Gasket (Bio-rad, USA). Oil droplets containing samples were generated using a QX200 Droplet generator (Bio-rad, USA). Sample droplets were carefully transferred into a 96-well PCR plate and sealed with Pierceable Foil Heat Seal (Bio-rad, USA) at 180ºC for 5 seconds. After that, thermal cycling was performed as follows:

1) 95.0 ºC 10:00 minutes
2) 94.0 ºC 0:30 minutes
3) 55.9 ºC 1:00 minute
4) Go to 2) for 39 more times
5) 98.0 ºC 10:00 minutes
6) 4.0 ºC Forever End

Heated lid and sample volume were set to 105ºC and 40 µL, respectively. Finally, the droplets were analyzed on a QX200™ droplet reader using QuantaSoft™ software. cccDNA copy was determined as an absolute count based on Poisson distribution calculated by the QuantaSoft™ program.

Statistical analysis

GraphPad Prism 7 software was used for correlation analysis of ddPCR detected cccDNA copies (practical value) compared to calculated cccDNA copies (theoretical value). A two-tailed p value of less than 0.05 was considered as statistically significant.

RESULTS

cccDNA specific primers optimization

In order to optimize the suitable annealing temperature for cccDNA primers and probes, gradient PCR was performed in qPCR and ddPCR. In qPCR, the temperature was varied from 50ºC to 60ºC. The results were shown in Figure 2 and Table 1, both of which present 58.3ºC as the temperature with the lowest quantification cycle (Cq) value. To confirm the specification of primers, PCR products, which have 322 bp, were run in gel electrophoresis. In every sample, the specific bands were presented between 300 – 400 bp, without any non-specific product, which can be seen in Figure 3. Therefore, 58.3ºC was indicated as the suitable annealing temperature for cccDNA primers and probes for qPCR thermal cycling.

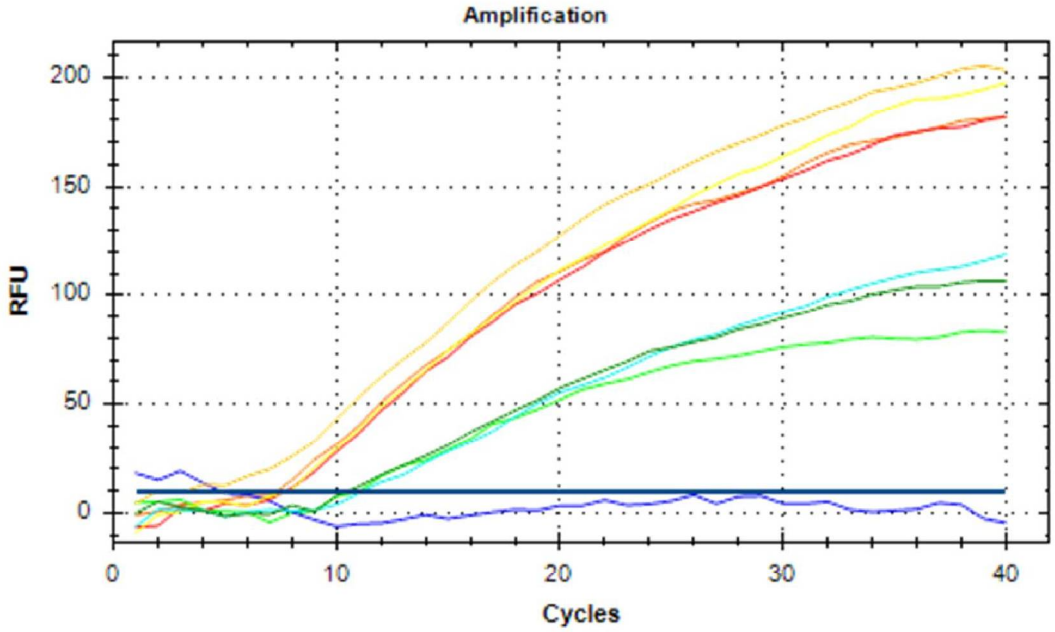

Figure 2. The Cq plot of gradient qPCR. The orange line (highest curve) demonstrates the annealing temperature at 58.3ºC, which presents the lowest Cq value. The red, dark orange, yellow, olive green, green, light blue, and blue colors represent the temperatures at 60.0, 59.4, 56.3, 53.9, 52.0, 50.7, and 50.0ºC, respectively.

Table 1. Comparison of Cq values from different annealing temperatures in gradient qPCR.

Fluorophore	Target	Content	Sample	Cq
FAM	cccDNA	Unknown	60.0	7.60
			59.4	7.25
			58.3	3.07
			56.3	7.57
			53.9	10.84
			52.0	10.35
			50.7	11.06
			50.0	N/A

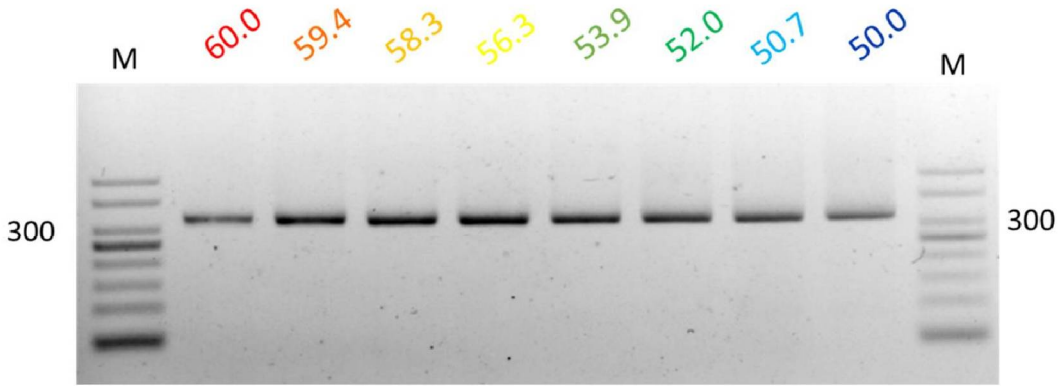

Figure 3. The specific bands amplified from cccDNA primers appear on agarose gel. M is a marker for the 50 bp ladders. The number on each lane indicates the annealing temperature that varied in gradient PCR.

In ddPCR, the annealing temperature was also graduated to verify the optimal temperature. Gradient PCR was performed by varying the temperature from 54ºC to 63. 2ºC. The 1- D amplitude graph generated from the QuantaSoft™ software demonstrated the improvement of the clear separation between positive (blue dots) and negative (gray dots) droplets when the temperature was increased as shown in Figure 4. The optimal temperature can be defined from the result as the one that expresses the largest fluorescence amplitude difference between positive and negative droplets and also avoids nonspecific amplification. From these criteria, 55.9ºC (lane F12) should be the optimal temperature for cccDNA primers in ddPCR experiment.

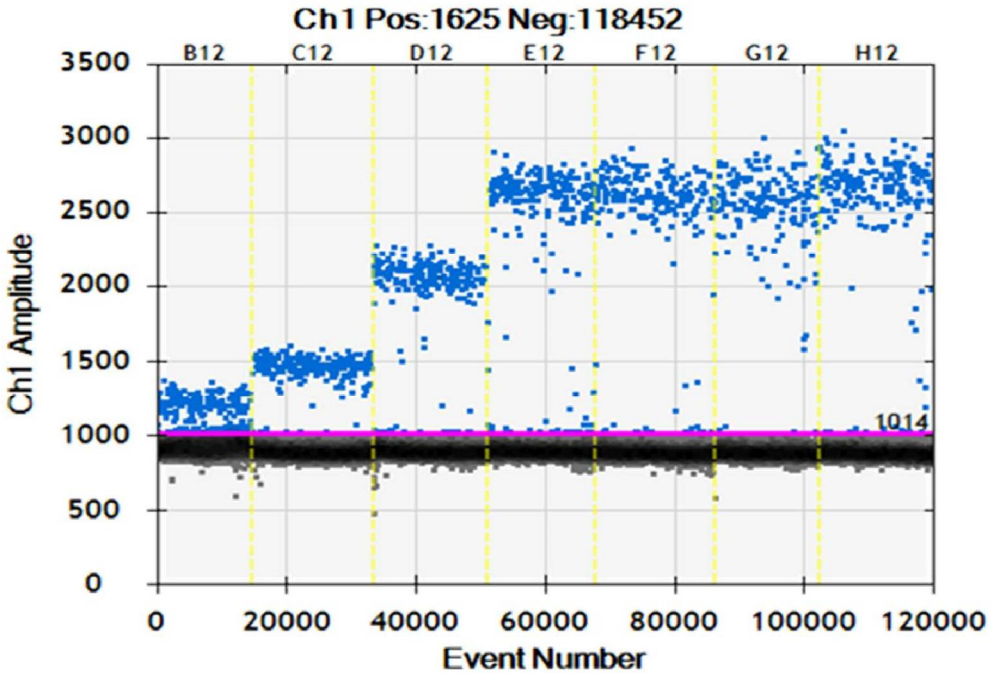

Figure 4. Thermal gradient optimization for cccDNA primers. Each lane from B12 to H12 indicates the temperature ordered from 63.2, 62, 60.1, 57.8, 55.9, 54.7, and 54ºC, respectively.

Sensitivity and accuracy of qPCR amplification system

To validate the sensitivity and accuracy of both PCR systems, the known concentration sample was used. In this experiment, the serial dilution from 10^{11} to 10^{-1} copies of HBV 1.3 mer WT plasmid were employed. However, this plasmid contains 2 targets for cccDNA primers, so the number of copies represented in results and used for calculation was 2×10^{11} to 2×10^{-1} copies of cccDNA targets instead of plasmid copies. The sensitivity of qPCR detection was presented in Figure 5. The relative fluorescence units (RFU) detected from the CFX96 Touch Real-Time PCR Detection System were plotted against cycles of amplification of each reaction that contained different concentrations of plasmid DNA template. The standard curve was constructed by converting the Cq value into the number of cccDNA targets using the equation: $\log N_o = \log N_f - C_q \log(1 + E)$, where N_o is the initial copy number of cccDNA target, N_f is the amplified copy number after Cq, and E is the efficiency of amplification. As shown in Figure 6, the copy numbers of cccDNA in log scale were demonstrated as an inverse linear relationship with Cq values following the equation: $y = 38.916 – 3.422x$, based on a coefficient

of determination (R^2) equal to 0.991, when the range of detection limit was 2×10^3 to 2×10^7 cccDNA copies. Even qPCR was able to detect cccDNA of more than 2×10^7 or less than 2×10^3 copies, but the R^2 and also the efficiency were dramatically reduced (data not shown).

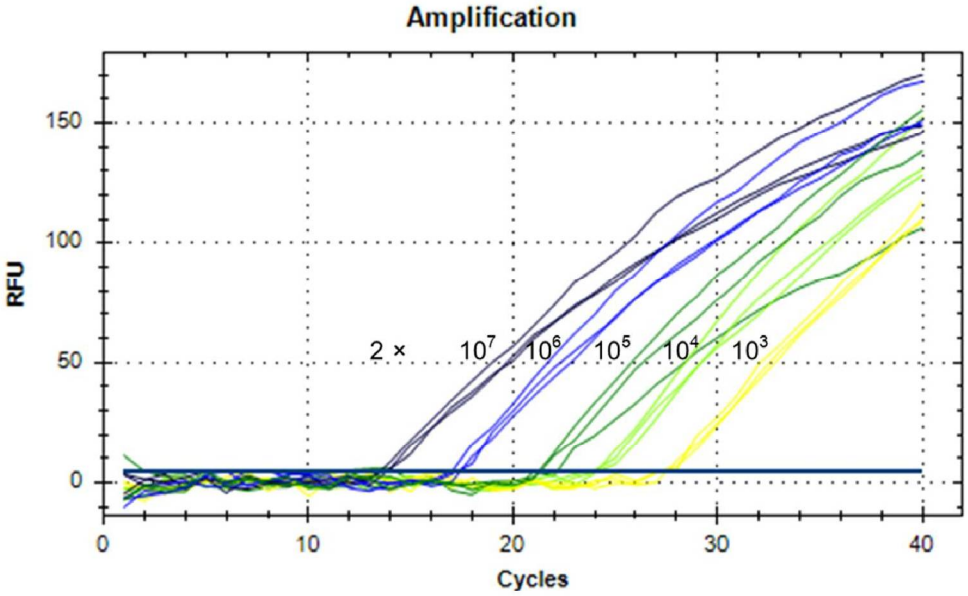

Figure 5. The sensitivity of qPCR to detected cccDNA target. Each number indicates the copies of cccDNA ranging from 2×10^7 to 2×10^3, that should be detected by qPCR.

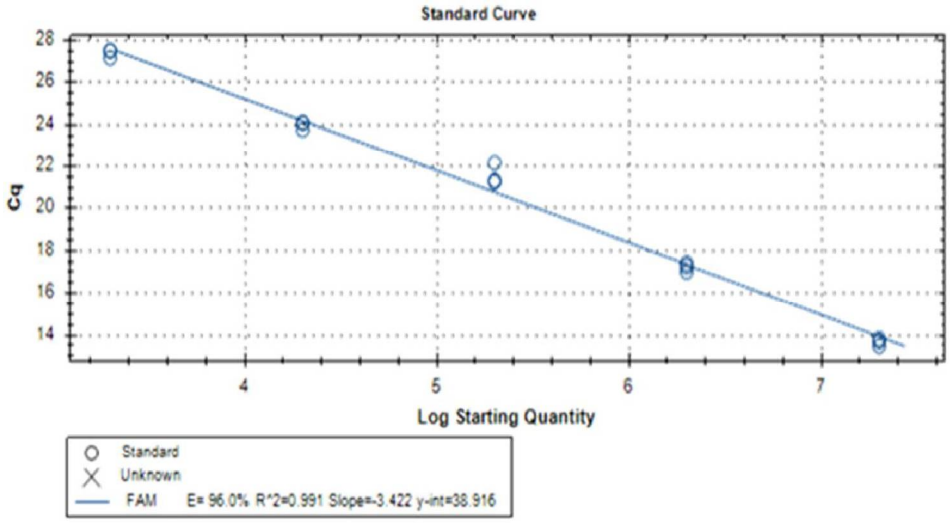

Figure 6. Quantification of serially diluted HBV 1.3 mer plasmid by qPCR. The values of amplification efficiency per cycle, slope of the fitted Line, and Y-intercept are shown as E, Slope, and y-int, respectively.

Sensitivity and accuracy of ddPCR amplification system

HBV 1.3 mer plasmid DNA was used as a template as it was in the qPCR assay. However, thermal cycling for ddPCR has to be performed when the PCR reactions are enclosed in droplets. HBV 1.3 mer plasmid is a circular DNA template which is difficult to contain in a droplet during the droplet generation process. Therefore, HBV 1.3 mer plasmid was pre-digested with BglII, which has its restriction sites located outside the cccDNA targets. After that, it was serially diluted from 2×10^{11} to 2×10^{-1} copies, as prepared in qPCR. Each concentration was applied to ddPCR reactions. The results are shown in Figure 7 as a dot plot graph plotting the copies/µL of the final 1X ddPCR reactions (Y-axis) for each cccDNA template copy.

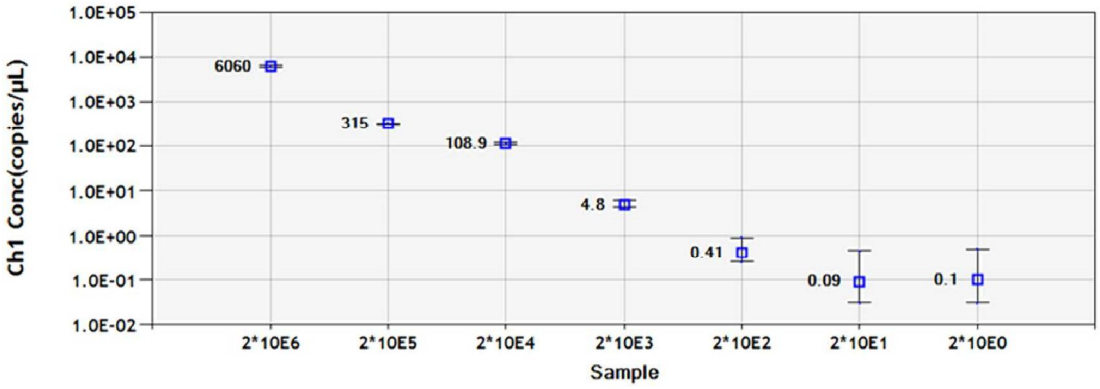

Figure 7. Detection of cccDNA using ddPCR. Each panel represents a cccDNA template copy in a ddPCR reaction. Y-axis represents the concentration of cccDNA targets in the final reactions.

In Figure 8, the serially diluted amounts of added DNA were plotted against the copies/ µL of cccDNA obtained from the calculation of QuantaSoft™ software as practical values. Moreover, each amount of DNA copy input was calculated for expected cccDNA copy output by dividing with the volume of the 1X ddPCR reaction. For example, 7.4×10^{-4} pg plasmid or 200 copies of cccDNA template were added into 20 µL of single ddPCR reaction. Therefore, the predicted result should be expressed as $200/20 = 10$ copies/µL in the final reaction. This calculation was applied to every cccDNA template concentration. As a result, the amounts of cccDNA input that ranged from 7.4×10^{-7} to 7.4×10^{5} pg were equal to 1.0×10^{-2} to 1.0×10^{10} copies/µL in the final reaction. These concentrations were plotted against the amount of DNA inputs as theoretical values. The Pearson correlation was calculated from GraphPad Prism 7 software and showed an R^2 value that was equal to 0.9966 ($p < 0.0001$) when the range of detection limit was around 1.0×10^{-1} to 1.0×10^{5} copies/µL (around 7.4×10^{-6} to 7.4 pg or 2 to 2×10^{6} copies put in the reactions). However, the quantification deteriorated at upper and lower levels of the cccDNA copies, which was not statistically significant (data not shown).

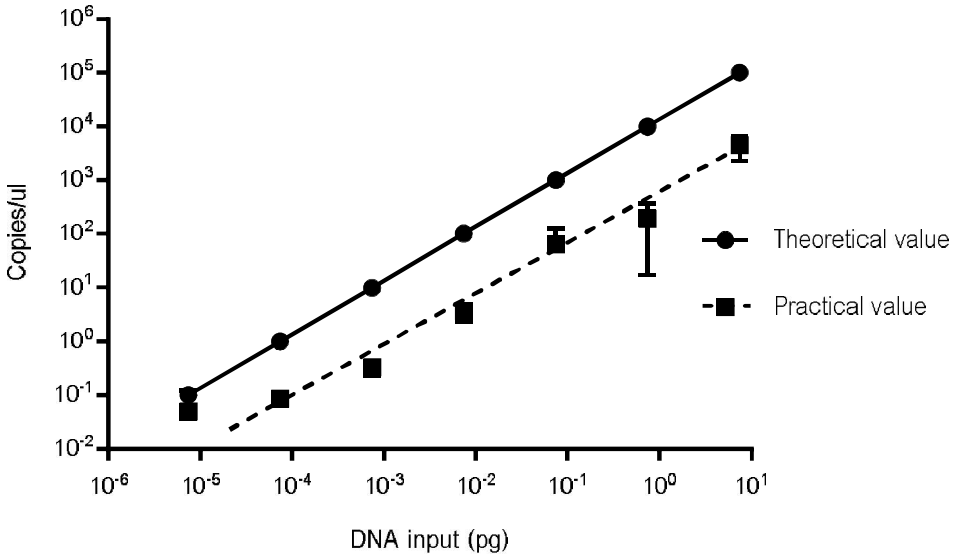

Figure 8. Differential sensitivity of ddPCR to detect cccDNA targets in HBV 1.3 mer plasmid. The theoretical values were converted from the amount of HBV 1.3 mer plasmid input by a formula of 2 copy of cccDNA target or 0.1 copies/µl in the final reaction = 7.4×10^{-6} pg plasmid DNA, while the practical values were the ddPCR-measured copy numbers.

DISCUSSION

Optimizing the annealing temperature is one of the most critical parameters determining PCR reaction specificity. Too low or too high annealing temperature might lead to nonspecific product amplification or reduction of desired PCR product yield (Rychlik, Spencer, and Rhoads, 1990). The annealing temperature using for ddPCR was lower than qPCR as stated by previous publications (Taylor et al., 2015; Brunetto et al., 2014). ddPCR presented the higher sensitivity of detection, at less than a single copy (Deprez et al., 2016), than qPCR. Nevertheless, the correlation between both methods was significantly strong when the number of DNA targets were increased (Strain et al, 2013) Moreover, ddPCR in this study demonstrated the larger dynamic range than previous (Hindson et al., 2011).

CONCLUSION

The novel ddPCR assay was successfully validated for applying to estimate cccDNA copy number. The optimal annealing temperature showed an improvement of the efficacy to amplify cccDNA target fragments by the largest separated distance between positive and negative droplets, with less nonspecific amplification. In addition, ddPCR showed the lowest detection limit around 0.1 copies/µL calculated by Poisson algorithm with high precision as $R^2 = 0.9966$ ($p < 0.0001$). However, qPCR might be the suitable method to detect a target DNA with high copies because its upper limit was higher than ddPCR. As cccDNA is presented in HBV infected cells at very low copies, ddPCR should be a platform for quantifying cccDNA. Prospectively, the performance of ddPCR for cccDNA copy estimation will be applied in subsequent researches in order to evaluate the precise cccDNA number after cccDNA-targeting treatments.

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