

# Comparison of a quantitative Real-Time PCR Assay and droplet digital PCR Assay for detection of *Streptococcus agalactiae*

Xu-Guang Guo (✉ [gysyngx@gmail.com](mailto:gysyngx@gmail.com))

Shaanxi Provincial People's Hospital

**Yi-Fan Zeng**

Institute of Kingmed of Guangzhou Medical University, Guangdong 510000, China

**Chu-Mao Chen**

Department of Clinical Laboratory Medicine, The Third Affiliated Hospital of Guangzhou Medicine University, Guangdong 510150, China

**Xiao-Yan Li**

Department of Laboratory Medicine, the Affiliated Shunde Hospital of Guangzhou Medical University, Foshan, China

**Jun-Jiang Chen**

Institute of Kingmed of Guangzhou Medical University, Guangdong 510000, China

**Yan-Ge Wang**

Institute of Kingmed of Guangzhou Medical University, Guangdong 510000, China

**Shi Ouyang**

Department of Infectious Disease, the Fifth Affiliated Hospital of Guangzhou Medical University, 510000, China

**Tian-Xing Ji**

Department of Clinical Medicine, the Second Affiliated Hospital of Guangzhou Medical University, 511436, China

**Yong Xia**

Department of Clinical Laboratory Medicine, the Third Affiliated Hospital of Guangzhou Medical University, Guangdong 510150, China

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## Research article

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# Abstract

**Background :** *Streptococcus agalactiae*(GBS) is pathogenic bacterium that causes puerperal sepsis in pregnant women and meningitis in newborns. Reliable detection methods for GBS included culture-based approaches, antigen-antibody method and real-time quantitative PCR. However, these methods have low sensitivity and are time consuming. In this study, we established a new detection approach, i.e. droplet digital PCR (ddPCR), for more accurate detection of GBS. **Materials and Methods :** Clinical specimens infected with GBS were analyzed using culture method. QPCR and ddPCR were used to quantify GBS related genes (CpsE and Sip gene). All experiments were run in triplicate using the same positive strain to verify the repeatability of the methodology. **Results:** The ddPCR showed outstanding accuracy, with 100% sensitivity, 100% specificity and coefficient of variation =4.5%. Among the detected strains, only positive results were obtained for the CpsE gene by ddPCR, and the detection sensitivity was 5pg/μL. Compared to the level of individual nanogramme-sized(0.5ng/μL) of qPCR assay, ddPCR resulted as a more significantly accurate method for detection of GBS in precision medical area. **Conclusions :** Droplet digital PCR offers high sensitivity, accuracy and repeatability, and is a suitable approach for detection of positive GBS samples.

## Introduction

*Streptococcus agalactiae* (Group B streptococcus, GBS) is a [gram-positive coccus](#) belonging to the [Lancefield classification](#) and is a kind of [catalase-negative](#), [β-hemolytic](#) and [facultative anaerobe](#). Despite GBS is a kind of harmless host bacteria considered as part of the human [microbiota](#), it colonizes the gastrointestinal and genitourinary tract, contributing to approximately 30% of the healthy adult as asymptomatic carriers in general<sup>1,2</sup>, which can lead to septicopyemia, pneumonia, meningitis through destroying host tissue and host immune mechanisms under special circumstances<sup>3,4</sup>.

GBS colonization in the gastrointestinal and genitourinary tract can sometimes cause serious problems for the mother and the newborn during pregnancy although it usually does not lead to serious illness in healthy women. The GBS infected mother can develop [chorioamnionitis](#) unusually-the possible cause of inducing premature delivery and the major cause of bacterial [infection](#) in newborns during pregnancy and after delivery with significant mortality rates in premature infants-and suffer from postpartum infections (after birth)<sup>4,5</sup>. The pathogenic factors of GBS include cell wall composition, extracellular enzyme and exotoxin, etc. Among the pathogenic factors it is generally accepted that β-haemolysin/cytolysin toxin which has ability of crossing the blood brain barrier plays an essential role in developing brain endothelial cells, neuronal lesions, so it takes responsibility of causing neonatal meningitis.

So far, most detection of GBS relied on culture-based approaches, antigen-antibody method, real-time quantitative PCR and so on. Since current culture methods are laborious and time consuming, and have limited sensitivity, antigen-antibody method has limited sensitivity, real-time qPCR needs standard curves to qualitative or quantitative test which is laborious and time consuming<sup>6</sup>. So more sensitive and quicker methods are needed in the clinical.

Here, we show that a GBS-special droplet digital PCR assay can detect significantly more accurate, precisely of GBS from clinical samples compared with methods of culture and real-time PCR individually. Droplet digital PCR uses oil-in-water technology to divide the mixtures into tens of thousands of nano-liters droplets. Each droplet is equivalent to an independent reaction system for amplification with or without nucleic acid target molecules. At the end of the reaction, the system calculates the absolute copy number of the original specimen according to the Poisson distribution. The quantitative results are no longer dependent on the CT value, and the initial concentration of the target sequence is given directly to achieve absolute quantization in essence<sup>7</sup>.

## Materials And Methods

### 2.1 Bacterial strains

Reference strain GBS ATCC13813 was purchased from Shanghai cell bank of Chinese Academy of Sciences. Other non-GBS bacteria that were used for sensitivity testing (*Candida tropicalis*, *Candida albicans*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, *Acinetobacter baumannii*, *Escherichia coli*, *Staphylococcus haemolyticus*, *Candida parapsilosis*, *Streptococcus anginosus*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*) were obtained from the Clinical Laboratory of Third Affiliated Hospital of Guangzhou Medical University. These bacteria were initially identified by Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry.

### 2.2 DNA Extraction

Each clinical specimen was inoculated into blood agar and cultured in 37°C constant thermostatic incubator for 18-24h to enrich bacterium. Consequently, bacteria were collected by swabbing; swabs were then mixed with physiological saline to obtain 1mL bacterial suspension. The bacterial DNA was then extracted using TIANGEN DNA purification kit (TIANGEN BIOTECH, Beijing) according to the manufacturer instructions, and was stored at -20°C until further analysis.

### 2.3 Primer design

Special primers were designed to detect sequences within the GBS *cpsE* gene and *sip* gene by Primer Premier5.0 (Premier Laboratories, Canada)<sup>5,8</sup>. The primer sequences were synthesized by Thermo Scientific of Shanghai Trade Co. Ltd (Table1).

### 2.4. Methods

#### 2.4.1 Real-time qPCR

Real-time Quantitative PCR assay was performed in a SLAN-96P-real time qPCR system (HONGSHI. Shanghai). Briefly, a total of 25μL reaction fluid was synthesized according to the kit instructions, under the following reactions: initial step of 95°C for 10min to denature; 45 cycles of 95°C for 15s and 60°C for 1 min to amplify nucleic acid.

## 2.4.2 Droplet Digital PCR

Droplet digital PCR was performed in a QX200 droplet digital PCR system (Bio-Rad Laboratories, CA). A total of 20ul reaction mixture, including enzymes, substrates, ions and DNA templates were transferred into the sample well of droplet generator cartridge (Bio-Rad). Then, a 70ul droplet oil was loaded into oil well. The droplet generator cartridge was then placed into the Droplet Generator to form water-in-oil droplets. Next, water-in-oil droplets were sucked from the well and transferred into 96-well PCR plate. The plate was sealed with foil to prevent aerosol pollution, and analyzed using Bulk PCR Thermal Cycler under the following conditions: 95°C for 10min to denature; 45 cycles of 95°C for 15s to destroy double-stranded DNA and 60°C for 1 min to amplify nucleic acid. Finally, after amplification reaction was loaded on a droplet reader. Whether the droplets can reflect a strong fluorescence depends on special amplifying of the gene sequences, which were designed for the GBS<sup>6,7,9,10</sup>. The results were interpreted based on the fluorescence signal intensity, the positive result was interpreted as “1” and the negative result was “0”.

## Results

### 3.1 Primer screening testing and specificity testing

Two primers were designed by Primer Premier 5.0 and used for quantitative PCR assay following the instructions. According to the request of reaction condition, we optimized the method, and then compared the amplification efficiency. The result curve indicated that CpsE sequences had good amplification efficiency compared to Sip sequences. The diagram information is shown in Figure 1. In addition, eleven non-GBS clinical isolations were tested by droplet digital PCR assay showing no amplification, which suggested that primer has a 100% specificity. The details are shown in Table 2.

### 3.2 Comparison of culture identification and molecular assays

Clinical GBS samples and non-GBS samples were selected from specimens of patients in the Clinical Laboratory of Third Affiliated Hospital of Guangzhou Medical University. Sixteen specimens were identified as GBS after pure culturing and were detected by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry<sup>11,12</sup>. Clinical isolates were analyzed by both droplet digital PCR and qPCR. These specimens were tested by droplet digital PCR and qPCR individually revealing that both droplet digital PCR and qPCR can test GBS and non-GBS correctly with 100% sensitivity and 100% specificity, which means that droplet digital PCR and qPCR are both reliable detection methods. The detailed data are shown in Table 2 and Table 3.

### 3.3 Sensitivity testing

Sensitivity between qPCR and droplet digital PCR were evaluated by using an array of dilutions of highly purified GBS DNA. The genomic DNA was diluted in normal saline to produce the concentrations between 5ng/μL and 0.05pg/μL<sup>13</sup>. The fluorescence of genomic amplification and the positive events after amplification reaction are shown in Figure 2 and 3, respectively. We found that ddPCR could identify

individual picogram-sized (5pg/μL) samples compared to quantitative PCR assays that could identify only individual nanogramme-sized (0.5ng/μL) samples (Figure 4), which suggested better sensitivity of ddPCR.

### **3.4 Reproducibility testing**

The mixture of GBS reference strains was run in triplicate. A negative control was used to examine the detection accuracy (Figure 5). The positive events were 1661, 1560 and 1704, their coefficient of variation (CV) was CV=4.5%, CV<5% which suggested that droplet digital PCR has an excellent reproducibility.

## **Discussion**

Droplet Digital PCR has several characteristics in detecting disease<sup>14</sup>. The advantages are following: 1) it allows for the precise quantification of nucleic acids without the need of standard curve. When the number of target DNA molecules is smaller than the number of microdrops, each droplet contains 0 or 1 target DNA, which means that the number of targeted DNA is equivalent to the positive microdrops. There are more than one target DNAs in some microdroplets when the number of target DNA molecules is high. Results analysis should comply to the theory that the number of target DNA number greater than positive microdrops. 2) ddPCR offers higher detection sensitivity compared to conventional PCR method. The sensitivity can reach a single nucleic acid molecule and the detection limit is low to 0.001%. The reason for that is the micro-droplet step can realize the enrichment of target DNA/RNA. 3) ddPCR has excellent repeatability. Improved accuracy and repeatability can be utilized to correctly determine the relative expression of target genes, gene copy number variation analysis, etc.<sup>15,16</sup>. Due to all these characteristics, ddPCR does not rely on Ct value and amplification efficiency, and can overcome the influence of the PCR inhibitors, i.e. it is suitable for analyzing blood, FFPE tissue, feces, urine, sputum, water, soil, plants and other complex samples.

Although ddPCR overcomes the difficulties of conventional PCR, it is also more prone to error when handled by inexperienced users. The major key factors that affect ddPCR are: 1) specificity of primers: because of its high accuracy, the existence of primer dimer can be revealed to a great extent, which will greatly interfere with the experimental results; 2) the procedure that loading 20μL reaction mixtures into the sample well of the droplet generator cartridge needs cautious operation. The method is absolutely not allowed bubbles in the reaction fluid, because the bubbles can gravely prevent the generation of water-in-oil droplets. If the total number of droplets is less than 12000, this experiment does not meet with the Poisson distribution, which means the experiment fails.

To sum up, our data suggested that ddPCR could be used as a method of choice for detection of GBS in clinical samples.

## **Declarations**

### **Ethics approval and consent to participate**

Not applicable.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

All data generated or analysed during this study are included in this published article.

### **Competing interests**

The authors declare that there are no competing interests associated with the manuscript.

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### **Authors' contributions**

YF Zeng, JJ Chen, XY Li and XG Guo conceived and designed the study. YF Zeng, JJ Chen and YG Wang cultured bacteria and carried out the ddPCR. CM Chen, S Ouyang and TX Ji extracted DNA and performed the Q-PCR. YF Zeng, JJ Chen, XY Li and CM Chen conducted data analysis to make figures and tables. YX Xia and XG Guo participated and gave guidance throughout the process. All members participated in the writing, review, discussion and revision of the manuscip and adopted the final version unanimously.

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## Tables

**Table1** Sequences of primers of sip and cpsE gene

target	Sequences(5'-3')
Sip upstream	CTGCCAACCACTATGACC
Sip downstream	CTGCTACAGTTCTTACCG
CpsE upstream	GCAAAAGAACAGATGGAACAAAGTG
CpsE downstream	CGCCGTAAGTAGCAACAGAT

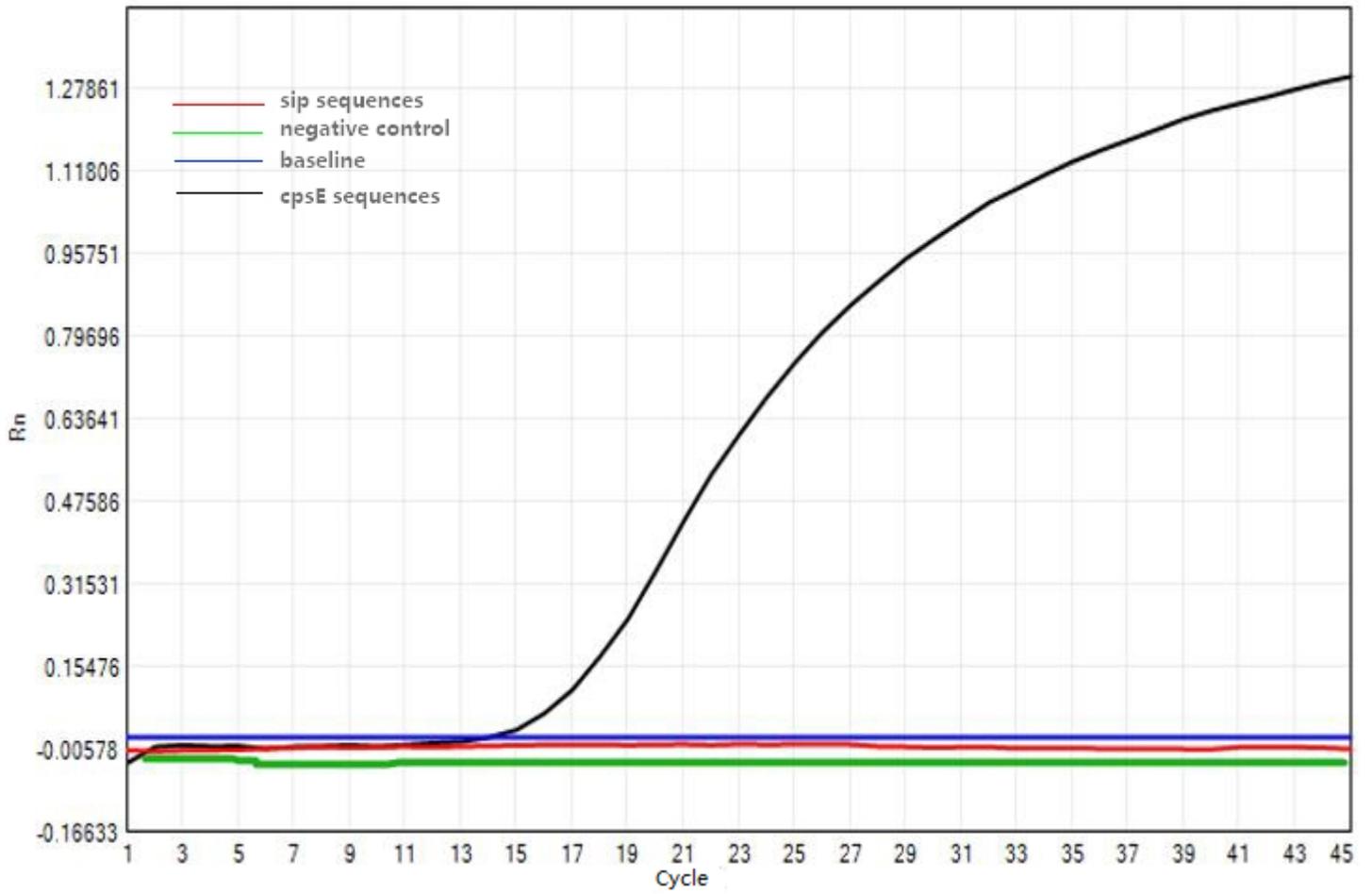
**Table 2** Analysis of cultured microbiology by droplet digital PCR

droplet digital PCR results	Culture test		
	GBS	non-GBS	
Positive	16	0	16
Negative	0	11	9
total	16	11	25
		Sensitivity=100%	Specificity=100%

**Table 3** Analysis of cultured microbiology by qPCR

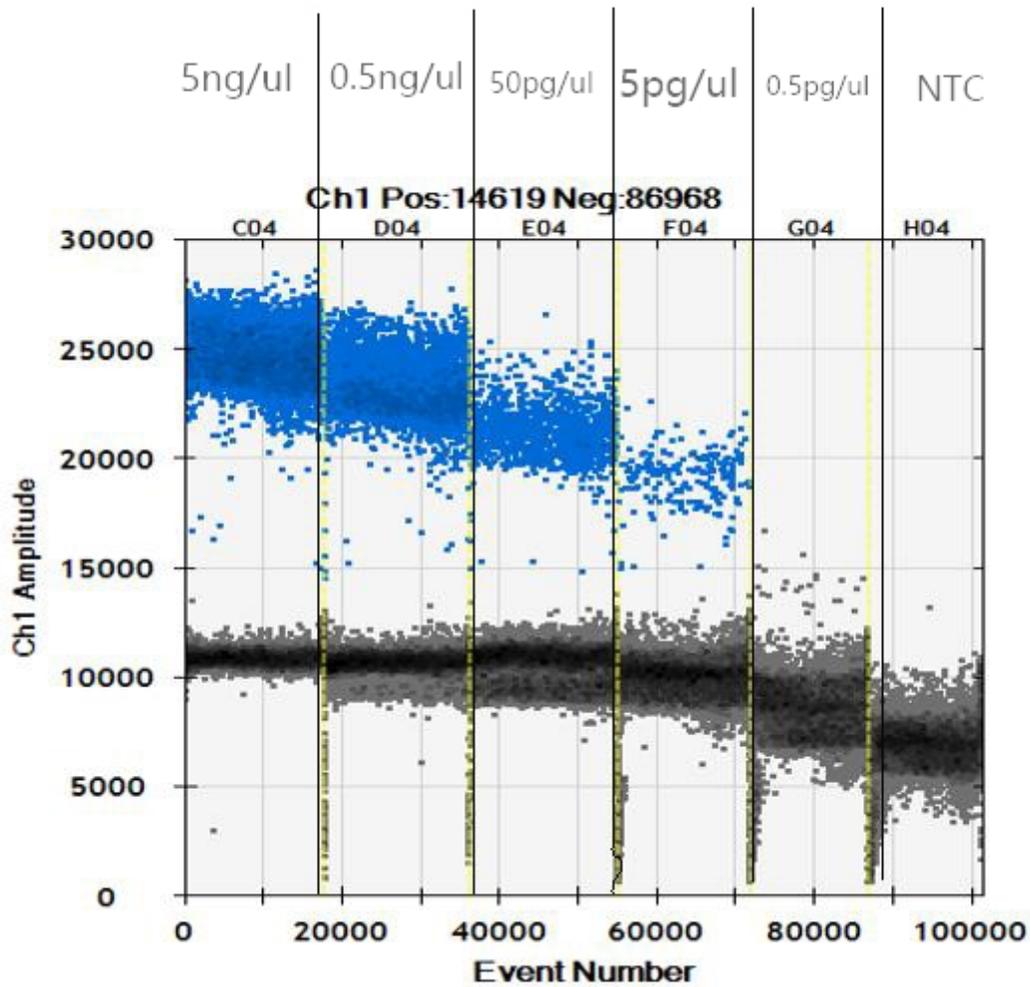
qPCR results	Culture test		
	GBS	non-GBS	
Positive	16	0	16
Negative	0	11	9
total	16	11	25
		Sensitivity=100%	Specificity=100%

## Figures



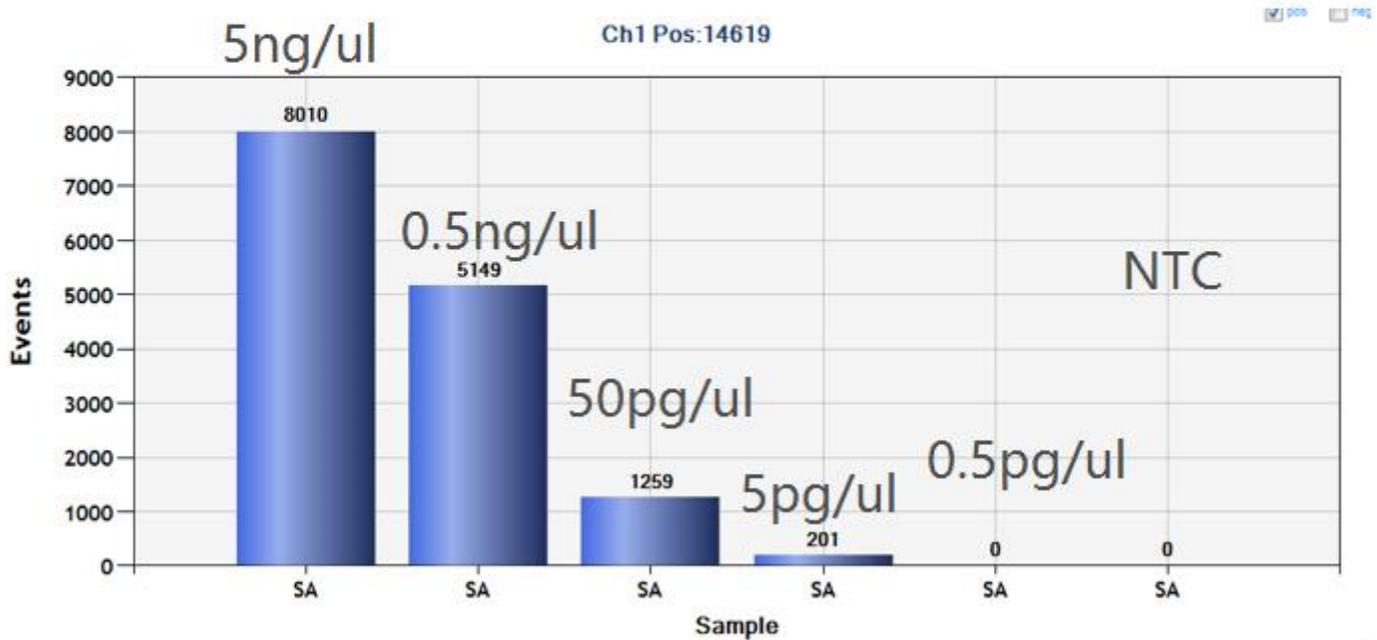
**Figure 1**

Primer screening testing and specificity testing results.



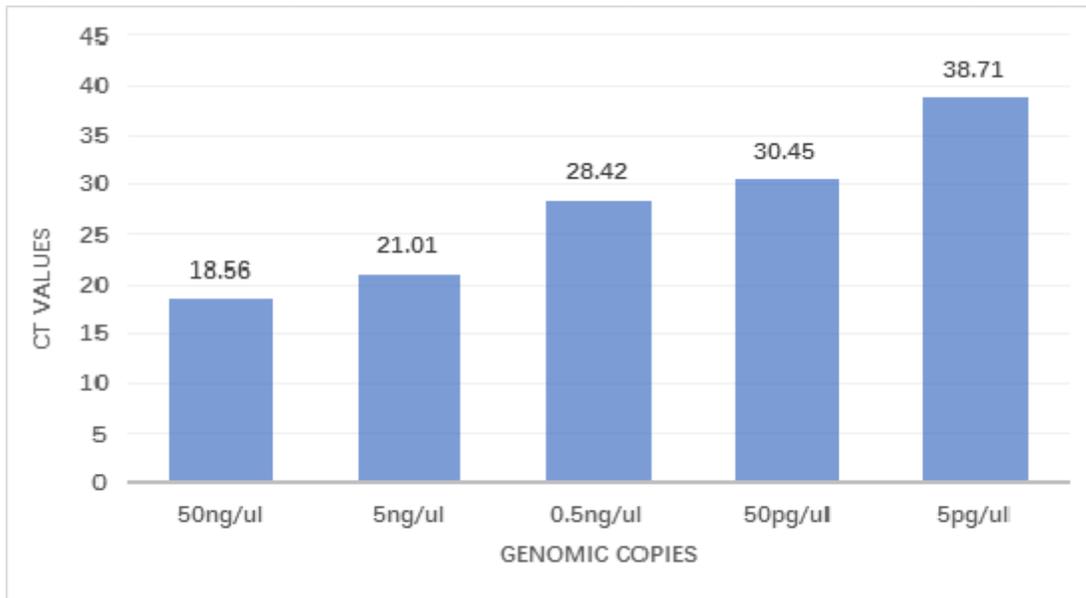
**Figure 2**

Representatives fluorescence of genomic amplification of droplet digital PCR assays for the genomic DNA which observed continuous 10-fold dilutions and negative control(NTC) was also included.



**Figure 3**

Representatives the positive events after amplification reaction of droplet digital PCR assays



**Figure 4**

Representative the quantitative PCR assays for the genomic DNA which observed continuous 10-fold dilutions.

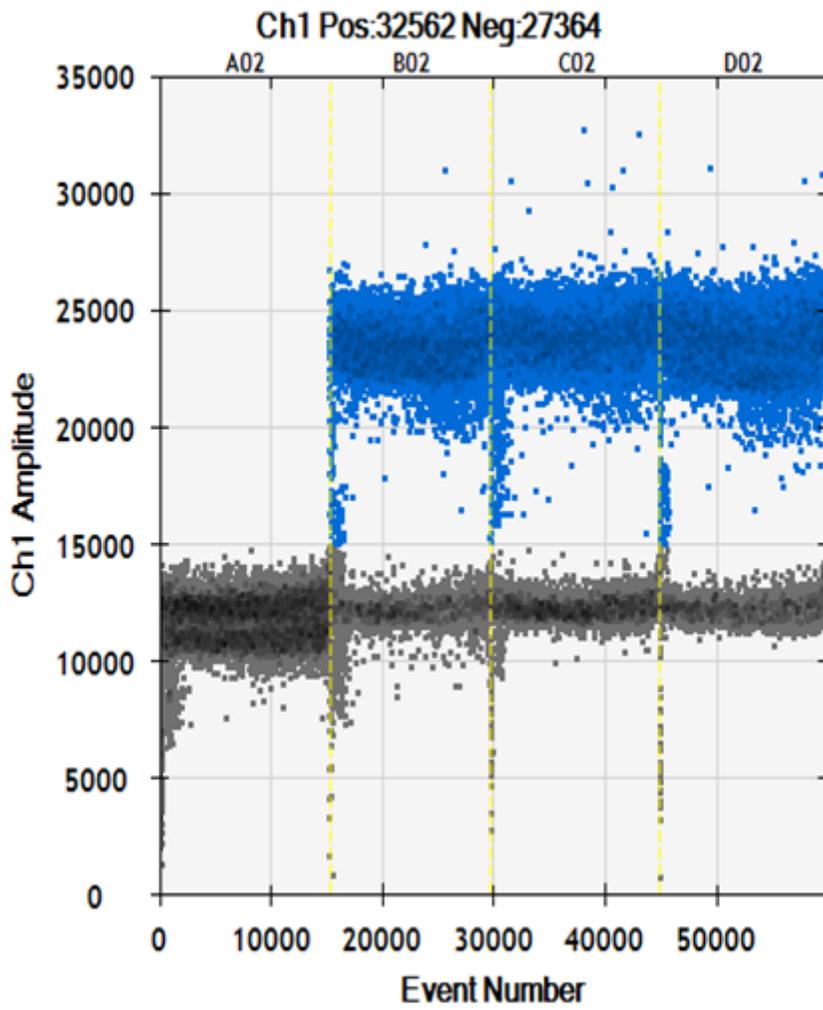


Figure 5

Representative the mixture of reference strains of GBS was run in triplicate and added a negative control.