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SHORT COMMUNICATION

# Development of a sensitive and reliable droplet digital PCR assay for the detection of '*Candidatus* Liberibacter asiaticus'

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**Abstract**

Citrus Huanglongbing (HLB, yellow shoot disease) is one of the most serious citrus diseases worldwide. To better improve the detection sensitivity, a droplet digital PCR (ddPCR) assay was developed for the rapid detection of '*Candidatus* Liberibacter asiaticus' (Las), the putative causal agent of HLB. The detection of sensitivity comparison using positive plasmid indicated that ddPCR was superior to quantitative PCR (qPCR) for detecting and quantifying Las at low concentrations. The Las detection of 40 field samples also showed that six of 13 asymptomatic samples (46.15%) with high $C_t$ value (>35) were positive by ddPCR. This methodology showed great potential for early HLB infection diagnosis.

**Keywords:** citrus Huanglongbing, early diagnosis, droplet digital PCR

## 1. Introduction

'*Candidatus* Liberibacter asiaticus' (Las), a phloem-resided α-protebacterium, is the putative causal agent of citrus Huanglongbing (HLB, yellow shoot disease) that is one of the most serious diseases in citrus production (Bové 2006). The bacterium is transmitted from infected to healthy plants through grafting or by citrus psyllid (*Diaphorina citri*). No effective cure is currently available for HLB-infected citrus plants. Therefore, the use of pathogen-free nursery stocks, control of insect vector and removal of infected trees are major control measures in HLB management. This is of particular importance if HLB infection status of asymptomatic trees in field could be accurately diagnosed for the implementation of control strategies.

Because Las is unable to be cultured so far, current detection is typically PCR-based using primers developed from genomic DNA sequences, mostly the 16S rRNA gene. Primer set OI1/OI2c for conventional PCR and primer-probe set HLBas/HLBp/HLBr for TaqMan real-time quantitative PCR (qPCR) are widely used for the standardized detection of Las (Jagoueix *et al*. 1994; Li *et al*. 2006). Recently, multi-copy genes have been chosen as targets for the improvement of qPCR sensitivity (Morgan *et al*. 2012; Zheng *et al*. 2016). However, absolute quantification of unculturable Las by qPCR is challenging due to erratic distribution and low titer, especially for early detection of Las infection.

Droplet digital PCR (ddPCR) is a new technology that allows sensitive detection and absolute quantification of low concentration DNA without the need for a standard curve.

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Each sample tested was partitioned in tens of thousands of individual droplets in a water-oil emulsion and then the number of positive droplets was read by cumulative fluorescence signal during PCR amplification. The total number of target DNA molecules in a sample can be calculated from the fraction of positive droplets and Poisson statistics (Hindson *et al*. 2011). Since ddPCR has been shown to yield more precise detection results than qPCR, the robust and powerful method has been increasingly used in medical researches (Taylor *et al*. 2015), clinical applications (Tsui *et al*. 2011; Watanabe *et al*. 2015), food safety inspection (Pinheiro *et al*. 2011; Floren *et al*. 2015) and gene-editing frequencies study (Mock *et al*. 2016). Recently, it also has been used to detect *Xanthomonas citri* subsp. *citri*, an economically important disease of citrus (Zhao *et al*. 2016).

In this study, we established ddPCR approach to detect and quantify Las in both symptomatic and asymptomatic samples. The detection sensitivity of ddPCR was compared to qPCR targeting the gene encoding 16S rRNA.

## 2. Materials and methods

### 2.1. Sample collection and DNA extraction

HLB symptomatic and asymptomatic field citrus samples were collected from Guangxi and Hunan of China. All collected samples in China were shipped by mail to Citrus Research Institute (CRI) of Southwest University in Chongqing, China. Four HLB-positive citrus samples and four negative citrus samples were collected from the greenhouse in CRI. The midribs of citrus leaves were excised and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) methods as previously described (Wang *et al*. 2012).

### 2.2. Preparation of cloned plasmid standard

A DNA segment encoding 16S rRNA gene of Las was amplified with Las genomic DNA as the template. The PCR amplicon was purified and ligated into the pEASY-T1 cloning vector (TransGen Biotech, China). Plasmid DNA was extracted from transformed competent cells and used to generate a standard curve for tenfold serial dilutions consisting of nine concentration gradients, which were used to test the sensitivities and linearity range of qPCR and ddPCR assays.

### 2.3. Quantitative PCR

The primers and probe targeted the 16S rRNA gene of Las were used in the subsequent qPCR and ddPCR assays (Li *et al*. 2006). The qPCR assay was performed on an iCyler IQ™ System (Bio-Rad, Hercules, CA, USA). The cycling conditions included incubation for 30 s at 95°C followed by 40 cycles of 95°C for 5 s and 58°C for 30 s. $C_t$ values were analyzed using BioRad iCycler iQ version 3.0 Software with auto-calculated baseline settings and a manually set threshold at 0.1. Standard curve was constructed through serial dilutions of plasmids for quantification and checked for qPCR efficiencies.

### 2.4. Droplet digital PCR

The QX200™ Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) was used in the study. The total ddPCR reaction volume was 20 μL, containing 10 μL 2× ddPCR™ supermix for probe (no dUTP) (Bio-Rad, Pleasanton, CA, USA), 1 μmol $L^{-1}$ of each primer, 500 nmol $L^{-1}$ of probe, and 2 μL template DNA. Approximately 20 000 droplets were generated using a Droplet Generator (DG) with an 8-channel DG8 cartridge and cartridge holder with 70 μL of DG oil per well and 20 μL of reaction mixture. Following this step, 40 μL droplets mixtures were transferred into a 96-well plate. The PCR plate was heat-sealed using a PX1™ PCR Plate Sealer (Bio-Rad) and placed in the C1000 Thermal Cycler (Bio-Rad) under the following thermal conditions (temperature ramp rate 2°C $s^{-1}$): 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 54°C for 1 min. Droplets were counted on the QX200 droplet reader (Bio-Rad).

### 2.5. Data analysis

Linear regression analyses of standard curve from qPCR was performed and recalculated with Microsoft Excel Software (Microsoft, USA). Slope value of standard curve was used to determine PCR efficiencies. For ddPCR, positive droplets were discriminated from negative droplets by applying a fluorescence amplitude threshold with the QuantaSoft™ version 1.7.4 (Bio-Rad). Correlation analysis between ddPCR and qPCR was performed with SPSS Software version 21.0 (SPSS Inc., Chicago, USA). Pearson's correlations and linear regression were also used to evaluate the relationship between measurements of ddPCR and qPCR assays.

## 3. Results and discussion

Adequate discrimination between positive and negative signals is of great importance to set appropriate thresholds. Annealing temperature conditions play important roles in determining fluorescence intensity and the distance between positive and negative signals. To assess the optimal annealing temperature of the ddPCR assay, the eight

temperature gradients ranged from 64 to 52°C were set on the thermal cycler. An optimized annealing temperature of 54°C was determined based on the largest discrimination in fluorescence intensity between positive and negative droplets.

To compare the linearity, dynamic range and sensitivity of qPCR and ddPCR assays, calibration curves for the qPCR assay and the regression curves for the ddPCR assay were constructed using tenfold serial dilutions of positive plasmid ($3.07\times10^{8}$–$3.07\times10^{1}$ copies $\mu L^{-1}$). Both qPCR and ddPCR assays exhibited good linearity of amplification with high determination coefficient ($R^2$=0.999 and 0.996, respectively) (Fig. 1-A and B). Furthermore, a very strong and significant positive correlation between the two methods ($r$=0.99; $P$<0.001) was observed (Fig. 1-C). The dynamic range tested in positive plasmid in qPCR was from $10^8$ to $10^2$. Compared to qPCR, ddPCR had the narrower linearity range from $10^5$ to $10^1$ copies since the droplets were positively saturated at target concentrations ≥$10^6$ copies $\mu L^{-1}$, making the Poisson algorithm invalid (Fig. 2-A). However, ddPCR showed a lower detection limit, suggesting the ddPCR is more sensitive than qPCR (Fig. 2).

The weak real-time PCR signals derived from low-concentration samples, as represented by high $C_t$ values, may be questionable for declaring a positive reaction. To better compare the detection sensitivity between the two assays, samples with $C_t$>35 tested by qPCR were regarded as Las-negative samples in this study. Total of 40 citrus samples extracted previously, with the $C_t$ value ranging from 28 to 38 by qPCR, were chosen for testing the detection capacity of ddPCR for high $C_t$ values samples and determining whether ddPCR can be used in the detection of field samples. Besides the relatively low $C_t$ value (<35) samples, six of 13 samples (46.15%) with high $C_t$ value (>35) were also positive by ddPCR (data not shown). It should be noted that asymptomatic citrus samples with low Las concentration could be detected by ddPCR, suggesting that ddPCR is a more robust method for the detection of samples with low concentration of Las, especially for samples in early infection and asymptomatic phase. The application of a microsimulation model of asymptomatic disease spread using psyllid introduction scenarious indicated that the surveillance and control should be used from the initial detection of invasion and throughout the asymptomatic period (Lee *et al*. 2015). The ddPCR-based technology will play an important role in the detection of Las from asymptomatic citrus samples. It is believed that if more primer pairs targeting multi-copy genes were used in ddPCR (Morgan *et al*. 2012; Zheng *et al*. 2016), the detection sensitivity might be improved accordingly.

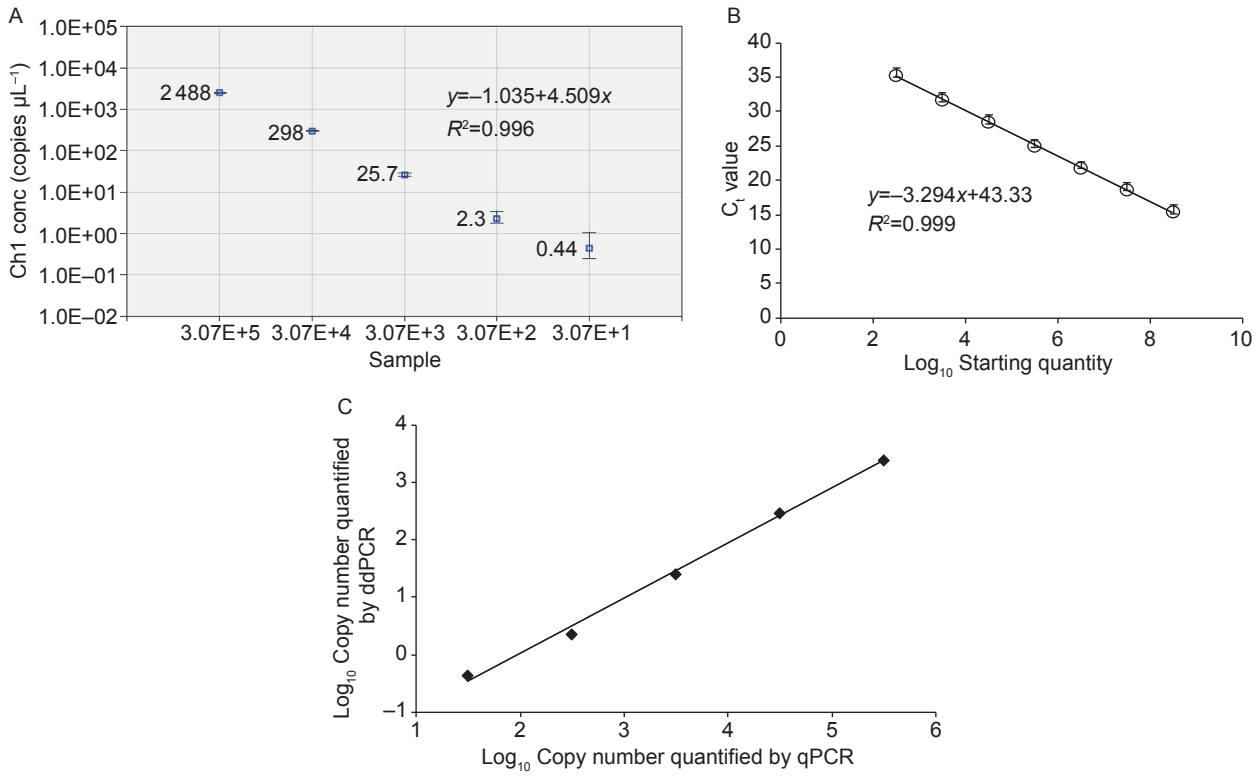


**Fig. 1** Linear regression of droplet digital PCR (ddPCR, A) and real-time quantitative PCR (qPCR) assays using serial tenfold dilutions of plasmid DNA (B), and correlation between $\log_{10}$ means of copies using ddPCR *vs*. qPCR (C). Data are means±SD (A and B).

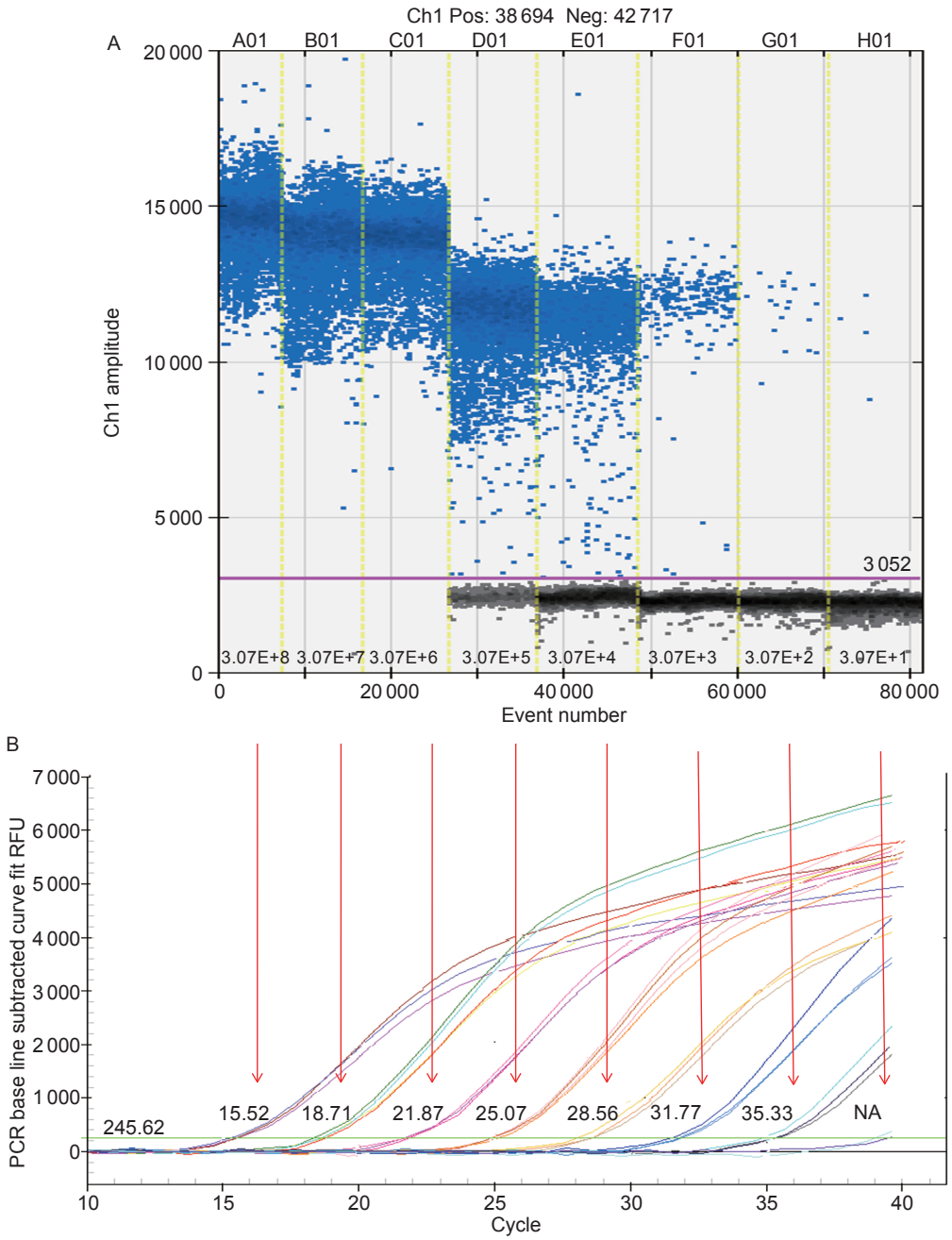


**Fig. 2** Sensitivity comparison of '*Candidatus* Liberibacter asiaticus' (Las) detection between droplet digital PCR (ddPCR, A) and real-time quantitative PCR (qPCR, B) assays. Eight ddPCR reactions with serially diluted targets are divided by the vertical dotted yellow line. The unbroken pink line is the threshold, above which are positive droplets (blue) containing the target DNA and below which are negative droplets (gray) without any target DNA. Each target concentration in ddPCR is corresponding to the $C_t$ value (from 15.52 to NA (not applicable) in qPCR by the red arrow. RFU, relative fluorescence units.

Recently, field-capable assays, loop mediated isothermal amplification (LAMP) and serologically based immune tissue print, have been developed for Las detection (Rigano *et al*. 2014; Ding *et al*. 2016, 2017). These methods offer the advantages of simplicity, low cost and high throughput in comparison with PCR-based assays currently used. However, uneven distribution and low titer of Las in citrus plants are still big challenges for these assays. The high sensitivity ddPCR assay could be an effective complement for the detection of early HLB infection or low titer samples.

## 4. Conclusion

This is the first report to demonstrate the ddPCR technology

for the quantification of Las. The detection sensitivity of ddPCR was compared to qPCR targeting the 16S rRNA gene. Our result showed that ddPCR was superior to qPCR for detecting and quantifying Las at low concentrations. Reducing risk of false negatives is critically important if PCR diagnosis of Las infection is used in certification programs. This methodology showed great potential for early HLB infection diagnosis.

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## References

Bové J M. 2006. Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. *Journal of Plant Pathology*, **88**, 7–37.

Ding F, Duan Y P, Yuan Q, Shao J, Hartung J S. 2016. Serological detection of "*Candidatus* Liberibacter asiaticus" in citrus, and identification by GeLC-MS/MS of a chaperone protein responding to cellular pathogens. *Scientific Reports*, **6**, 29272.

Ding F, Paul C, Brlansky R, Hartung J S. 2017. Immune tissue print and immune capture-PCR for diagnosis and detection of *Candidatus* Liberibacter asiaticus. *Scientific Reports*, **7**, 46467.

Floren C, Wiedemann I, Brenig B, Schütz E, Beck J. 2015. Species identification and quantification in meat and meat products using droplet digital PCR (ddPCR). *Food Chemistry*, **173**, 1054–1058.

Hindson B J, Ness K D, Masquelier D A, Belgrader P, Heredia N J, Makarewicz A J, Bright I J, Lucero M Y, Hiddessen A L, Legler T C. 2011. High-throughput droplet digital PCR system for absolute quantitation of DNA copy number. *Analytical Chemistry*, **83**, 8604–8610.

Jagoueix S, Bove J M, Garnier M. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the α subdivision of the *Proteobacteria*. *International Journal of Systematic and Evolutionary Microbiology*, **44**, 379–386.

Lee J A, Halbert S E, Dawson W O, Robertson C J, Keesling J E, Singer B H. 2015. Asymptomatic spread of Huanglongbing and implications for disease control. *Proceedings of the National Academy of Sciences of the United States of America*, **112**, 7605–7610.

Li W, Hartung J S, Levy L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. *Journal of Microbiological Methods*, **66**, 104–115.

Mock U, Hauber I, Fehse B. 2016. Digital PCR to assess gene-editing frequencies (GEF-dPCR) mediated by designer nucleases. *Nature Protocols*, **11**, 598–615.

Morgan J K, Zhou L, Li W, Shatters R G, Keremane M, Duan Y P. 2012. Improved real-time PCR detection of '*Candidatus* Liberibacter asiaticus' from citrus and psyllid hosts by targeting the intragenic tandem-repeats of its prophage genes. *Molecular and Cellular Probes*, **26**, 90–98.

Pinheiro L B, Coleman V A, Hindson C M, Herrmann J, Hindson B J, Bhat S, Emslie K R. 2011. Evaluation of a droplet digital polymerase chain reaction format for DNA copy number quantification. *Analytical Chemistry*, **84**, 1003–1011.

Rigano L A, Malamud F, Orce I G, Filippone M P, Marano M R, Morais do Amaral A, Castagnaro A P, Vojnov A A. 2014. Rapid and sensitive detection of *Candidatus* Liberibacter asiaticus by loop mediated isothermal amplication combined with a lateral flow dipstick. *BMC Microbiology*, **14**, 86.

Taylor S C, Carbonneau J, Shelton D N, Boivin G. 2015. Optimization of droplet digital PCR from RNA and DNA extracts with direct comparison to RT-qPCR: Clinical implications for quantification of Oseltamivir-resistant subpopulations. *Journal of Virological Methods*, **224**, 58–66.

Tsui N B, Kadir R A, Chan K A, Chi C, Mellars G, Tuddenham E G, Leung T Y, Lau T K, Chiu R W, Lo Y D. 2011. Noninvasive prenatal diagnosis of hemophilia by microfluidics digital PCR analysis of maternal plasma DNA. *Blood*, **117**, 3684–3691.

Wang X, Zhou C, Deng X, Su H, Chen J. 2012. Molecular characterization of a mosaic locus in the genome of '*Candidatus* Liberibacter asiaticus'. *BMC Microbiology*, **12**, 18.

Watanabe M, Kawaguchi T, Isa S I, Ando M, Tamiya A, Kubo A, Saka H, Takeo S, Adachi H, Tagawa T. 2015. Ultra-sensitive detection of the pretreatment EGFR T790M mutation in non-small cell lung cancer patients with an EGFR-activating mutation using droplet digital PCR. *Clinical Cancer Research*, **21**, 3552–3560.

Zhao Y, Xia Q, Yin Y, Wang Z. 2016. Comparison of droplet digital PCR and quantitative PCR assays for quantitative detection of *Xanthomonas citri* Subsp. *citri*. *PLoS ONE*, **11**, e0159004.

Zheng Z, Xu M, Bao M, Wu F, Chen J, Deng X. 2016. Unusual five copies and dual forms of nrdB in "*Candidatus* Liberibacter asiaticus": Biological implications and PCR detection application. *Scientific Reports*, **6**, doi: 10.1038/srep39020

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