

# Integration of microfluidic chip with Loop-mediated Isothermal Amplification (LAMP) assay for rapid quantification of *Enterococcus faecalis*

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## ABSTRACT

Rapid and sensitive monitoring of water quality can effectively prevent accidental human exposure to waterborne pathogens. In this project, a rapid quantification method was developed for *Enterococcus faecalis*, a fecal indicator bacterium (FIB), used as a surrogate for water quality assurance. The loop-mediated isothermal amplification (LAMP) assay was carried out on a microfluidic chip to produce thousands of individual reactions that convert the qualitative LAMP assay to quantitative results based on Poisson distribution. The isothermal amplification removes the need of thermal cycling, which makes it suitable for field deployment. In this assay, the water sample is mixed with LAMP reagents in the presence of fluorescent dye. The mixture is then dispersed into thousands of droplets encapsulated within the oil phase using a microfluidic droplet generator chip, where each droplet acts as an individual LAMP reaction. By counting the number of positive droplets among total droplets in the viewing field, the most probable number of the *E. faecalis* can be quantified statistically. The optimized oil phase can generate monodisperse droplets of 55  $\mu\text{m}$  in diameter, which were stable during LAMP reaction at 65°C for 30 mins. The limit of detection of droplet LAMP assay is 4 CFU/ reaction in pure culture.

**Keywords:** LAMP, microfluidics, *Enterococcus faecalis*, water quality, pathogen indicator quantification

## 1 INTRODUCTION

The global burden of waterborne disease owing to unsafe drinking water, inadequate or non-existent sanitation and hygiene is a major challenge to public health and can be associated with significant financial burden[1]. Natural or manmade disasters may also deteriorate aqueous environments and trigger locally waterborne-disease outbreaks. For example, massive power outage as a common side effect of devastating disasters may cause wastewater treatment plant malfunction, which may lead to the discharge of untreated or inadequately treated sewage to the environment. The disease-causing pathogens carried by human sewage may further spread and contaminate aqueous environment and drinking water sources. Therefore, a field-applicable microbial pathogen diag-

nostic system is critically needed for evaluating the local microbial contamination level, especially in resource-limited regions and post-disaster situations. However, the deployment of current pathogen detection technologies is limited by the requirement of dedicated laboratory facilities equipped with clean water, power and well-trained personnel. Such requirements are hindering the timely data collection and public health protection.

Microfluidic technologies have gained attention in improving the performance of molecular diagnostic assays and applicability of point-of-care diagnostic devices. The integration between polymerase chain reaction (PCR) and microfluidic technologies offers advantages over conventional PCR assay by providing better quantitative results with improved precision and reproducibility[2]. For example, the droplet digital PCR (ddPCR) system from Bio-Rad is capable of quantification of microbial pathogens within a few hours. However, it consists of a series of sophisticated instruments that function separately, which makes it cost-prohibitive for onsite water quality assessments in resource-limited regions. Therefore, it is necessary to develop alternative system for rapid, sensitive, and cost-efficient detection and quantification of waterborne pathogens. Loop-mediated isothermal amplification (LAMP) is an emerging DNA amplification method that can amplify DNA under constant temperature (65°C-72°C [2]). A set of 6 primers recognizing 8 distinct regions of target DNA provides LAMP higher specificity than PCR assay. However, LAMP only yields binary results (i.e. positive or negative). The combination of LAMP binary outcome and microfluidic droplet system can convert traditional LAMP to a quantitative method.

In this project, a microfluidic chip based droplet LAMP method was developed for the simultaneous detection and quantification of microbial pathogens. *Enterococcus faecalis*, a fecal indicator bacterium, was used as a model microorganism for method demonstration.

## 2 MATERIAL AND METHODS

### 2.1 Sample Preparation

*Enterococcus faecalis* (ATCC 29212) was used for evaluating the sensitivity of droplet LAMP assay. Before each LAMP test, a fresh overnight *E. faecalis* liq-

uid culture was prepared. The applicability of droplet LAMP assay was evaluated using *E. faecalis* spiked water samples, including seawater, San Diego creek water and secondary effluent from Orange County Sanitation District.

LAMP primer sets designed previously [3] for targeting *azoA* gene of *E. faecalis* were employed in this study. To prepare the LAMP reaction mix, DNA sample was crudely extracted using bead-beating method. A total of 200  $\mu\text{l}$  of each *E. faecalis* spiked water sample was transferred into sterile screw-cap Eppendorf tubes with the addition of 0.5 g of 0.5 mm silica beads (BioSpec Products, OK, USA). Subsequently, the cells were disrupted using vortex mixer at the maximum speed for 6 min to obtain crude DNA extracts from the cells. After the centrifugation under  $10,000 \times g$  for 1 min, the supernatant was recovered as the DNA template. For LAMP reaction *E. faecalis* DNA sample was denatured by heating at  $85^\circ\text{C}$  for 2 min and immediately placed on ice. 2  $\mu\text{l}$  of denatured DNA sample was mixed with LAMP reagents according to the protocol of WarmStart LAMP Kit (New England Biolabs (NEB), MA, USA) and bovine serum albumin (BSA) (1  $\mu\text{g}/\mu\text{l}$ ) was added to reduce the adsorption of DNA and polymerase to droplet surface.

## 2.2 Microfluidic Chip Design and Fabrication

The microfluidic chip design used in this study is similar to that previously designed by Hatch, Andrew C., et al for droplet based digital PCR. In this study, PCR reaction was replaced with LAMP reaction to simplify the experimental procedure [4]. A flow-focusing geometry was used for initial parent droplet ( $\approx 11 \text{ nl}$ ) generation. A repeating bifurcation design (bifurcation junctions with a bifurcation angle of  $45^\circ$ ) with seven consecutive splitting of parent droplet produces 128 daughter droplets from a single parent droplet (Figure 5). The combination of flow-focusing droplet generation and consecutive bifurcation junctions ensures robustly generation of large quantity of monodispersed droplet and high frequency of LAMP reaction sample encapsulation.

A glass-polydimethylsiloxane (PDMS)-glass sandwich structure was used to minimize the adverse impact of air permeability of PDMS on droplet stability, as shown in Figure 2. Two glass slides served as effective permeation barriers for sealing the PDMS chamber from both sides. The PDMS-glass hybrid microfluidic chips were fabricated through standard soft lithography processes.

## 2.3 Microfluidic Chip Operation

LAMP reaction sample encapsulation was achieved using heavy mineral oil (Sigma-Aldrich) with 3% w/w Abil EM 90 (Degussa/Goldschmidt) and 0.1% w/w Triton X-100 (Sigma-Aldrich) as stabilizing surfactants. To

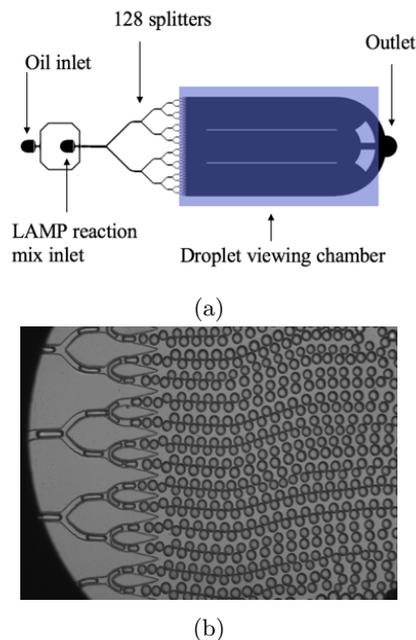


Figure 1: (a) Schematic illustration of microfluidic chip design. (b) Consecutive droplet fission.

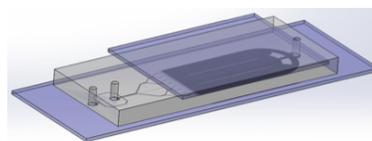


Figure 2: Schematic view of the droplet LAMP chip. Top and bottom layers were glass slides and the middle layer was patterned PDMS.

generate droplets, fresh prepared LAMP reaction mixture and oil were stored in two screw-cap tubes, where microbore tubings (Tygon, Sigma) were mounted to ensure fluidic connection between off-chip sample reservoirs and microfluidic device. External pneumatic pressure pump controlled by a scalable pressure modulator was used to inject LAMP reaction sample and oil into the chip from two inlets respectively. For droplet generation, the operational pressure of aqueous and oil phases was set for 1.6 psi and 2.6 psi, respectively, resulting in the droplet generation frequency of 0.384kHz and 61.5% water/oil (w/o) volume ratio. Subsequently, generated droplets were collected in a  $12.8\text{mm} \times 19.2\text{mm}$  droplet viewing chamber. When the chamber was filled with droplets, the chip was placed on a heating block and incubated at  $65^\circ\text{C}$  for 30 min. After incubation, microscopic observations of fluorescent droplets were made using Olympus IX71 fluorescence microscope, the fluorescent droplets were imaged using a high resolution monochrome cooled CCD camera (ORCA-D2, Hamamatsu). An image processing software ImageJ (NIH) was used to measure droplet size, analysis fluorescence

intensity, and quantify fluorescent droplets. The concentration of *E. faecalis* in LAMP sample could be calculated statistically according to the following equation:

$$C = -\frac{\ln(1 - \frac{N_p}{N_{total}})}{V_{droplet}}$$

where  $N_{total}$  is the total number of droplets in each image,  $N_p$  is the number of positive droplets, and  $N_p/N_{total}$  is the fraction of positive droplets,  $V_{droplet}$  is the droplet volume ( $\mu\text{l}$ ).

### 3 RESULTS AND DISCUSSION

#### 3.1 Sensitivity of droplet LAMP

The sensitivity of droplet LAMP assay was evaluated using the DNA sample extracted from a ten-fold serial dilution of *E. faecalis* pure culture (the corresponding *E. faecalis* concentration ranged from with 4 to  $4 \times 10^4$  CFU/reaction). The *E. faecalis* concentration calculated using droplet data was compared with viable plate counts for droplet LAMP data validation. The representative images of droplet LAMP chip after incubation were shown in Figure 3. As the *E. faecalis* concentration increases, a corresponding increase in the percentage of LAMP positive droplets could be observed.

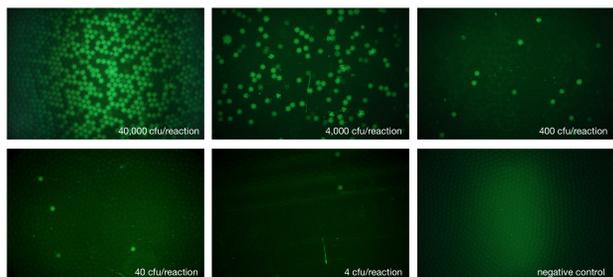


Figure 3: Droplet LAMP results with a serial dilution of *E. faecalis* ranging from 4 to  $4 \times 10^4$  CFU/reaction.

The lower detection limit of droplet LAMP was near the order of 4 CFU/reaction in *E. faecalis* pure culture. The droplet LAMP results showed a positive log linear correlation ( $R^2=0.9819$ ) between the concentration of *E. faecalis* derived from droplet LAMP and that from culture method. The discrepancy occurred at the high concentration may related to the limitation on the dynamic range of the droplet assay as the upper limit of detection of droplet LAMP is determined by the total number of droplet generated per reaction. The relatively large error at the lower concentration range may be due to the loss of DNA sample during DNA preparation. Furthermore, droplet image selection bias may also contribute to the variation between the calculated and expected values. Image capture of large field of view is essential to the accuracy and precision of droplet data

analysis when a relative large number of droplet could be captured each time. Because of the limitation of cameras field of view, multiple regions within the chamber were imaged.

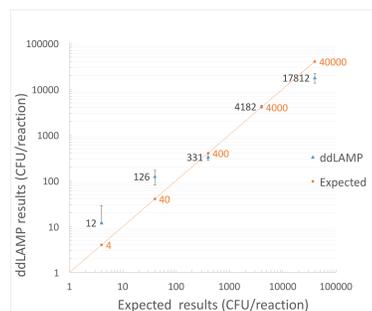


Figure 4: Comparison of the droplet LAMP results and viable plate counts (data was plotted on logarithmic scales).

A bias could be introduced when combining the quantification data from several captured images. For an expected concentration of 4 CFU/reaction, one of the selected region may show 2 positive droplets, which would provide a relatively higher percentage of positive droplet. However, the real percentage of positive droplet for the entire chamber may be much lower. Since the fraction of positive droplet is positively correlated to the calculated sample concentration, the variation on the ratio of positive to total number of droplet may further affect the accuracy of sample concentration calculation. Therefore, the degree of image overlap and number of image captured within each reaction played important roles in droplet data analysis.

#### 3.2 Field Application of droplet LAMP assay

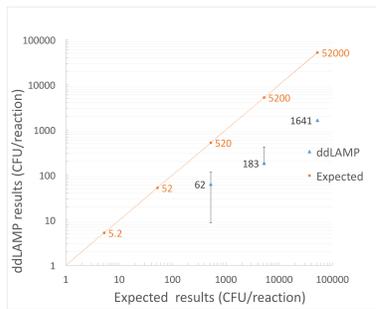
The applicability of droplet LAMP assay was evaluated using three different environmental water samples. The collected water samples were spiked with *E. faecalis* due to the low *E. faecalis* concentration in original water sample. The DNA samples extracted from ten-fold serial dilutions of each type of spiked water sample were first tested in triplicates using conventional LAMP method in test tubes, and then quantified using droplet LAMP assay. Table 1 shows the sensitivity tests performed with traditional LAMP. LAMP results were positive in all water samples, and the lower limit of detection was around 40 CFU/reaction which was an order of magnitude lower than that in *E. faecalis* pure culture.

The results show that the performance of LAMP varies from sample to sample. The availability and detection limit of LAMP decreased as the complexity of sample matrix increased. Since DNA sample preparation mainly relied on mechanical disruption of cells (i.e. bead beating and centrifugation), LAMP inhibitors in

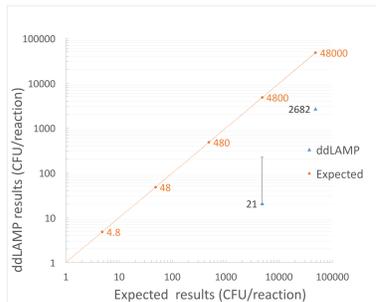
Water samples	E.faecalis concentration (CFU/reaction)				
	40,000	4,000	400	40	4
<i>E.faecalis</i> pure culture	3(3)	3(3)	3(3)	3(3)	3(3)
<i>E.faecalis</i> spiked seawater	2(3)	1(3)	0(3)	0(3)	0(3)
<i>E.faecalis</i> spiked San Diego creek water	3(3)	3(3)	1(3)	1(3)	0(3)
<i>E.faecalis</i> spiked secondary effluent	3(3)	3(3)	1(3)	0(3)	0(3)

Table 1: Results of the sensitivity tests performed with traditional LAMP assay

sample matrix are not be efficiently removed from DNA sample. To some extent, the presence of impurities in crude DNA extracts sample may affect the enzymatic activities and therefore hinder the assay performance and effectiveness in certain events. In addition, the DNA amplification efficiency and sensitivity of LAMP assay could also be affected by the quantity of DNA templates recovered. Since the DNA recovery was subject to other impurity and the particle surfaces including the silica beads, the quantity of DNA template varies based on water matrices. Moreover, ionic strength of water sample may alter the affinity between silica beads and DNA molecules and therefore the recovery of DNA in the supernatant. In spite of the lower sensitivity in comparison with the pure water, there is a strong positive correlation between starting concentration of cells and quantification by LAMP reaction (Table 1 and Figure 5).



(a) *E.faecalis* spiked OCSD secondary effluent



(b) *E.faecalis* spiked San Diego Creek water

Figure 5: The performance of droplet LAMP on samples of different matrices

Figure 5 shows the performance of droplet LAMP on *E. faecalis* spiked environmental samples. In compari-

son with the quantification results of *E. faecalis* pure culture, droplet LAMP showed a comparatively narrower effective range when high-complexity environmental matrices were introduced in reaction mixture. Valid droplet quantification data could not be obtained when a lower concentration sample was used. The asynchronous change of the percentage of positive droplet and sample concentration may be because of the persistence of LAMP inhibitor in extracted DNA sample or the undesired template DNA loss during DNA sample preparation. Therefore, to improve the sensitivity and applicability of droplet LAMP, further DNA purification should be conducted after mechanical cell lysis.

## 4 CONCLUSION

In this study, we have demonstrated a promising approach for the rapid, specific, and quantitative detection of *Enterococcus faecalis*, using a droplet LAMP assay on a microfluidic device. The combination of heavy mineral oil with 3% Abil EM 90 and 0.1% Triton X-100 shows high chemical compatibility with different environmental water samples. The glass-PDMS-glass chip configuration effectively maintains droplet integrity during on-chip amplification. The addition of fluorescent dye allows the visual discrimination between LAMP positive and negative droplets, and works in conjunction with droplet image recognition software to achieve a semi-automated conversion from fluorescence signal to quantitative droplet data. Although the sensitivity of droplet LAMP quantifying target microorganism in water sample is subject to the purity of DNA sample and the cameras field of view at current stage, the droplet LAMP assay still shows promise to become a rapid diagnostic tool in the field of pathogen indicators and pathogen detection and quantification.

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