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Absolute Quantification of H5-Subtype Avian Influenza Viruses Using Droplet Digital Loop-Mediated Isothermal Amplification

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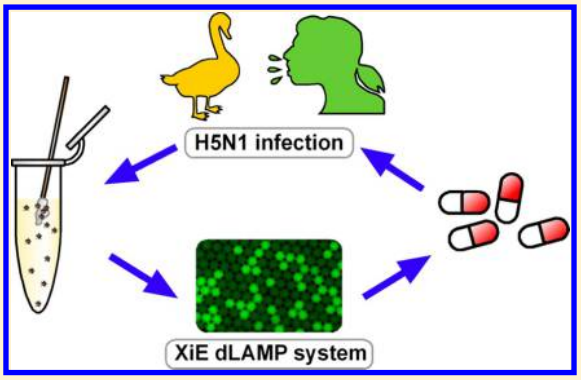
Supporting Information

ABSTRACT: Human infection with avian influenza A H5N1 viruses can cause severe diseases with high mortality rate and continues to pose a significant threat to global public health. Rapid diagnosis is needed for identifying the types of influenza viruses for making timely treatment decisions. Here, we demonstrate absolute quantification of H5-subtype influenza viruses by digital loop-mediated isothermal amplification (dLAMP) on our recently developed cross-interface emulsification (XiE) method. Our results show that XiE-based dLAMP is highly specific and displays comparable sensitivity to real-time PCR (qPCR) and digital PCR (dPCR). Notably, dLAMP is more tolerant to inhibitory substances than PCR methods and demonstrated similar detection efficiency to qPCR for real H5N1 samples. Therefore, it can serve as a robust and precise alternative to qPCR or dPCR and is especially suitable for environmental and clinical samples with hard-to-remove contaminants. We believe that our dLAMP method offers great potential for rapid and accurate diagnosis of influenza and other infectious diseases.



This paper describes rapid viral load quantification of highly pathogenic avian influenza (HPAI) infections based on digital loop-mediated isothermal amplification (dLAMP), using our recently developed cross-interface emulsification (XiE) system. HPAI H5N1 viruses are deadly for wild birds and poultry and have caused severe human diseases with fatal outcome since the first human case reported in 1997.[1,2] Sporadic outbreaks of HPAI are not expected to diminish in the short term, posing a continuing threat to global public health. Rapid, specific, and quantitative detection of HPAI is especially important for infection monitoring and control, evaluating virus-host interactions, and antiviral medications.[3]

Among various influenza diagnosis methods, real-time quantitative polymerase chain reaction (qPCR) has been commonly accepted for its exceptional sensitivity and specificity with rapid turnaround time, approaching 2 h.[4,5] However, it requires highly trained personnel, expensive instruments, and extensive hands-on time. Also, efficiency of qPCR is greatly impaired when samples contain inhibitory substances,[6] which brings up increasing concerns about accuracy and robustness of qPCR. Digital PCR (dPCR) marks a significant step forward which measures absolute quantities by counting nucleic acids partitioned in a large number of microvolumes.[7−11] It is independent of standards, more tolerant to inhibitors, and has been widely used for both research and clinical applications.[12−14] Following dPCR, various digital isothermal amplification methods are recently emerging,[15−18] which further eradicate the need for expensive thermal cyclers and hold great potential for point-of-care use. Among these methods, digital loop-mediated isothermal amplification (dLAMP) arouses great interests because of its high robustness, sensitivity, and specificity. Several microfluidic devices have been reported for dLAMP, including continuous flow device,[19] sample self-digitization chip,[20] SlipChip,[21] and so forth. These devices have greatly expanded the recognition of dLAMP as an excellent alternative to dPCR. However, limited work has been done with regard to the viral load quantification using dLAMP, and the requirement of expensive microfabrication techniques has prevented further implementation of dLAMP in rapid HPAI diagnostics, especially in resource limited settings. So far, there is still much more work to be done to establish simple, rapid, and cost-effective viral load quantification methods for HPAI H5N1, as well as other fatal viruses such as HPAI H7N9,[22] Ebola,[23] and Zika.[24]

We recently introduced cross-interface emulsification (XiE),[25] a simple method to produce droplets with tunable volume from tens of picoliters to nanoliters. XiE generates monodisperse droplets using a vibrating capillary, which can be easily set up and is user-friendly to those who lack microfabrication facilities. Additionally, thousands of droplets can be precisely controlled to form planar monolayer droplet arrays (PMDA) in flat-bottomed containers for reaction and detection. We validated that XiE could serve as a simple and

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robust platform for digital amplification of nucleic acids such as dLAMP and dPCR.

In this study, we performed XiE-based dLAMP assays for absolute quantification of H5-subtype HPAI viruses. dLAMP was highly specific and sensitive with a detection limit of less than 10 copies per reaction and was highly tolerant to inhibitory substances. With regard to quantification of H5N1 viral loads from real samples, dLAMP and qPCR showed comparable detection efficiency. We believe this simple and low-cost dLAMP approach offers great opportunity for rapid viral loads quantification of HPAI and other infectious pathogens.

EXPERIMENTAL SECTION

RNA Extraction and Reverse Transcription. Influenza A virus H5N1 (A/environment/Qinghai/1/2008), H1N1 (A/Hangzhou/05/2009), H3N2 (A/swine/Guangxi/07/2005), and H7N9 (A/chicken/Hangzhou/174/2013) used in this study were stored in the lab. We extracted total viral RNA using Trizol (Thermo Scientific, Waltham, WA) according to the manufacturer's instructions. The total RNA was then reverse transcribed into cDNA by the GoScript Reverse Transcription System (Promega, Madison, WI).

Primer Sequences. The hemagglutinin (HA) gene sequence information for the H5-subtype influenza A virus was obtained from the Global Initiative on Sharing Avian Influenza Data (GISAID) database and GenBank. The LAMP primers were designed by the Primer Explorer version 4 (https://primerexplorer.jp) and synthesized by BGI (ShenZhen, China). These primer sequences are H5−F3 (5′-3′): GAGTAATGGAAATTTCATTG; H5−B3 (5′-3′): CGCAAGGACTAATCTGTTTGA; H5-FIP (5′-3′): TGCAGTTACCATATTCCAATTCACTCAGAAAATGCATACA-AAATTGTC; H5-BIP (5′-3′): AACTCCAATAGGGGCGATAAACTCTTCACATATTTGGGGCATTC; H5-LF (5′-3′): ATAATTGTTGAGTCCCCTTTC; H5-LB (5′-3′): ATGCCATTCCACAACATCCAC. H5−F3 and H5−B3 primers were also used in qPCR and dPCR for comparison.

Preparation of qPCR Standards. The target HA gene was amplified from H5N1 cDNA using H5-F3 and H5-B3 primers by PCR. The product was purified and ligated into the pEASY-T1 vector using pEASY-T1 Simple Cloning Kit (Transgen Biotech, Beijing, China) following the protocol provided by the manufacturer. The ligation product was transferred into the Trans1-T1 Phage Resistant Chemically Competent Cell (Transgen Biotech, Beijing, China). Plasmids were extracted from PCR-verified positive colonies, and the concentration was determined by NanoDrop (Thermo Scientific, Waltham, WA). 10-Fold serial diluted plasmid samples were used to make the standard curve.

LAMP. Each LAMP reaction was in a 25 μL system containing 2.5 μL of 10 × ThermoPol buffer (New England Biolabs), 1.6 μM each of H5-FIP and H5-BIP primers, 0.2 μM each of H5-F3 and H5-B3, 0.8 μM each of H5-LF and H5-LB, 8 U of the Bst DNA polymerase (New England Biolabs, Ipswich, MA), 1.4 mM of each dNTPs (New England Biolabs, Ipswich, MA), 0.6 M betaine (Sigma-Aldrich, St. Louis, MO), 6 mM MgSO4 (Sigma-Aldrich, St. Louis, MO), 25 μM of Calcein (Sigma-Aldrich, St. Louis, MO), 0.5 mM MnCl2, 2 mg/mL BSA (New England Biolabs, Ipswich, MA), 1 μL of the template, and ddH_2O. Reaction tubes were incubated at 62 °C for 1 h, and results were displayed by direct visualization of the color change of solutions. Positive reactions are light green, and negative reactions are orange. Every experiment included a nontemplate control and was conducted in triplicates.

dLAMP on the XiE Platform. For dLAMP, we loaded the premixed LAMP reaction mixtures into the XiE platform and generated droplet arrays in a flat-bottomed 96-well plate as described previously.[25] Briefly, the capillary was vibrating across the oil surface contained in wells of a flat-bottomed 96-well plate at a frequency of 50 Hz (Figure S1). We infuse the LAMP reaction solution through the capillary at a constant flow rate of 10−200 nL/s with a syringe pump. For 1 nL droplets, we set a target volume of 2.5 μL per well to generate 2500 droplets to form PMDAs. For 0.2 nL and 4 nL droplets, the target volumes were 1 and 4 μL to obtain PMDAs composed of 5000 and 1000 droplets per well, respectively. We incubated the plate with the PMDAs at 62 °C for 1 h and obtained fluorescence images of the PMDAs with an inverted fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan). We analyzed the images and counted positive droplets with ImageJ (NIH) software and then calculated the template concentrations according to the Poisson distribution.[26]

qPCR and dPCR. The qPCR reaction system contained: 5 μL 2 × iTaq Universal Probes Supermix (Bio-Rad, Hercules, CA); 0.5 μL (10 μM each) H5-F3 and H5-B3; 0.25 μL (10 μM) H5-probe (5′FAM-CGATAAACTCTAGTATGCCATTCCACAACA-3′BHQ1); 1 μL template, and ddH_2O to a total volume of 10 μL. Nontemplate controls were set using ddH_2O as the template. The optimal cycling program was 95 °C for 30 s, 40 cycles of 95 °C for 15 s, 57 °C for 30 s, and storage at 4 °C.

The dPCR reaction system contained: 10 μL 2× QX200 ddPCR Supermix for Probes (Bio-Rad, Hercules, CA); 0.5 μL (10 μM each) H5-F3 and H5-B3; 0.25 μL (10 μM) H5-probe; 1 μL template, and ddH_2O to a total volume of 20 μL. Negative controls were set using ddH_2O as the template. The reaction mixtures were loaded into the QX200 system (Bio-Rad, Hercules, CA) for droplet generation. Droplets were then transferred to a 96-well plate and put into a thermal cycler. The cycling program was 95 °C for 10 min, 40 cycles of 95 °C for 30 s and 57 °C for 1 min, and then 98 °C for 10 min, storage at 4 °C. The thermal cycler ramping rate was set as 2 °C/s. After amplification, the plate was put into the QX200 reader to collect droplet signals. Data were analyzed using QuantaSoft (Bio-Rad, Hercules, CA), and the threshold for each reaction was set individually according to the nontemplate control.

Quantification of Viral Loads for Allantoic Fluid Samples. Ten nasopharyngeal swab samples were collected from H5N1-infected wild birds and inoculated in the allantoic cavity of 10-day-old embryonated hens' eggs at 37 °C for 72 h. All samples were stored in the −80 °C freezer and thawed in ice when used. Two hundred microliters of the allantoic fluids were used for the RNA extraction by the Trizol method. GoScript Reverse Transcription System (Promega, Madison, WI) was used for the reverse-transcription. One μL cDNA of each sample was used as a template for dLAMP and qPCR performed in parallel.

RESULTS AND DISCUSSION

Workflow. We recently introduced a simple and robust XiE method to perform dLAMP.[25] In this work, we applied dLAMP for absolute quantification of HPAI viral loads and compared its performance with qPCR and dPCR in parallel (Figure 1a). The common step is to isolate viral RNA from samples followed by reverse-transcription to obtain cDNA used as templates added

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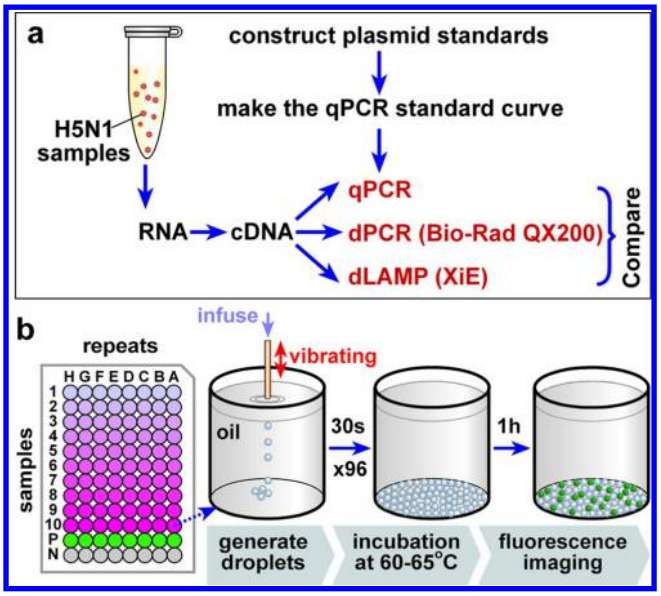

Figure 1. (a) Workflow of the absolute quantification of viral loads using real-time PCR (qPCR), digital PCR (dPCR) on Bio-Rad QX200, and digital LAMP (dLAMP) on the cross-interface emulsification (XiE) system. (b) Brief process of dLAMP, including generation of droplet arrays in microwells, incubation at 60−65 °C, and fluorescence imaging.

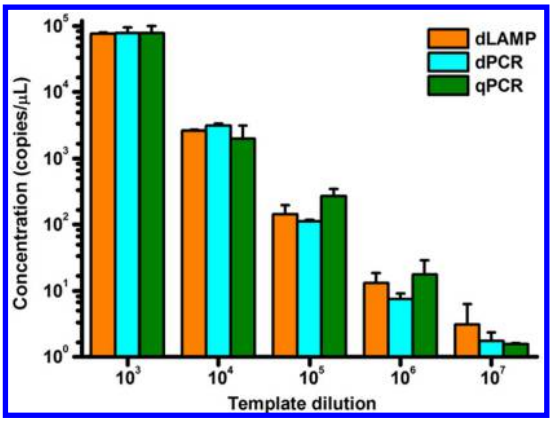

Figure 2. Comparison of dLAMP with different methods in detection of highly pathogenic avian influenza (HPAI) H5N1 viruses. 10-Fold serial-diluted H5N1 cDNA samples were used as the templates for qPCR, dLAMP, and dPCR in parallel.

to the Mastermix for all three methods. qPCR requires extra efforts to make the standard curve every time when performing quantification. The procedure of dLAMP includes (Figure 1b): (1) generation of droplet arrays in the flat-bottomed wells of a 96-well plate using the XiE system; (2) isothermal incubation of the plate in a 60−65 °C oven for amplification; and (3) fluorescence imaging of droplet arrays and data analysis.

To improve the dynamic range and accuracy of dLAMP, we used multiple microwells for each sample to produce and analyze 20000 droplets of multivolume,[27] from 0.2 nL (for high concentration) to 4 nL (for low concentration). XiE-based dLAMP has the following advantages: (1) LAMP reaction is faster than PCR-based assays and favorable for rapid diagnostics;[28] (2) dLAMP is independent of standard curves compared with qPCR; (3) compared with other droplet-based dPCR platforms, our method avoids droplets transfer and loss by using the same flat-bottomed well plate for droplet generation, amplification, and imaging. Moreover, we can set up dLAMP on many 96-well plates simultaneously in a single 60−65 °C oven with no need of expensive thermal cyclers, which is especially suitable for analyzing a large number of samples with limited resources.

Performance Comparison Among dLAMP, dPCR, and qPCR. We evaluated the performance of dLAMP using 10-fold serial diluted templates, which were also used as standards in qPCR. For quantitative assays by qPCR, dLAMP, and dPCR, all three methods obtained comparable results with detection limits of less than 10 copies/μL (Figure 2, Figure S2). It has been reported that viral loads in human specimens are in the range of $10^{4.3}$−$10^{8.2}$ copies/mL,[29] which is consistent with the dynamic range (10^4−10^8 copies/mL) we tested. Notably, all three methods showed a larger decrease in measured concentration for a 10-fold dilution of templates (slope < −1) (Figure S2), meaning we obtained fewer copies of templates from our experiments than theoretical calculation. The reason was likely due to template loss (by pipetting errors or template degradation) during serial dilution.

qPCR is currently regarded as the "gold standard" in analysis of nucleic acids, it relies on standard curves made by the coamplification of standard samples, which in this study are plasmids containing the target sequence. However, there may be a significant variation of the amplification efficiency between the standards and samples to be tested. The nucleic acid copy number of standard samples is calculated based on the concentration determined by the spectrophotometer, which might overestimate the real copy number of the templates. Therefore, digital nucleic acids amplification by dPCR and dLAMP is more attractive for their simplicity in quantification and high accuracy.

On the basis of our experience, dPCR on QX200 still has some potential problems that limit its performance: (1) droplet generation, PCR amplification, and signal collection are performed in different containers, requiring different corresponding machines. This multistep operation introduces inevitable droplet loss during droplet transfer, which results in less template detected and introduce technical variation among replicates. (2) It has a fixed protocol and reaction system, including the reagents and reaction volume, which limits researchers to optimize the assays based on their own needs or recipes. (3) As previously reported,[30] the fluorescence threshold of droplets is set subjectively, and signals of positive and negative events are sometimes not well-separated, making it difficult to adjust the threshold (Figure S3b). Our solution to this problem is to include nontemplate controls in every assay and set the threshold according to the nontemplate control. And three or more replicates are required for each assay to obtain more accurate quantification results. Additionally, performing dPCR using probes can significantly improve the specificity and accuracy than using Evagreen fluorescence dye.

XiE-based dLAMP method addressed these problems by simplifying droplet manipulation and using more robust LAMP chemistry. Once droplets were generated by XiE, all the subsequent manipulation steps were within the same container without droplet transfer, minimizing reagent loss and hands-on time. The ratio between positive and negative events could be measured accurately due to the evident difference (approximate 3-fold) of fluorescence signals between positive and negative droplets.[25] In addition, we could load reaction mixtures of different volumes into the capillary and generate droplets of

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different volumes to expend the dynamic range of quantification. Therefore, XiE-based dLAMP demonstrated excellent accuracy, reliability, and flexibility in the quantification of nuclei acids.

Specificity of dLAMP. To test the specificity of dLAMP, we used cDNA templates derived from representative influenza viruses belonging to H1, H3, H5, and H7 subtypes. In each test, we added the same amount of templates of either a single subtype or a combination of different subtypes by dLAMP. As shown in Figure 3, when using templates of a single subtype,

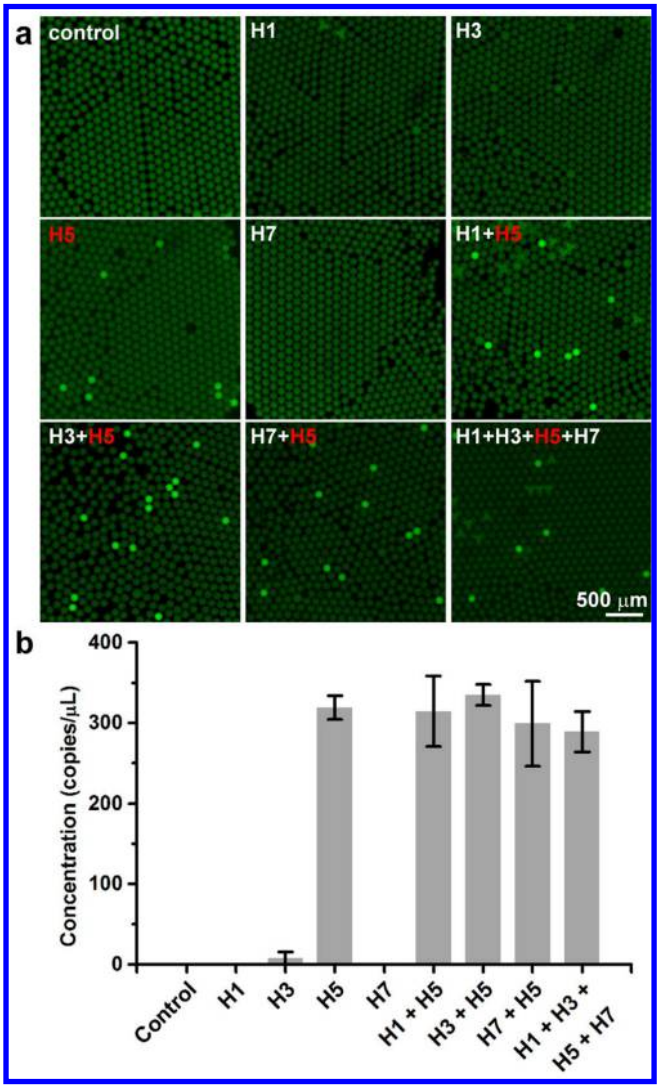

Figure 3. (a) Typical fluorescence imaging of droplet arrays after the dLAMP reaction. Viral cDNA of different subtypes added in each assay as templates were labeled on the upper left of each image. There was no template introduced in the control. (b) Evaluation of specificity of dLAMP assay in detection of H5N1 viruses.

dLAMP only detected the H5-subtype template but not the other three templates, yielding a concentration of 320 copies/μL. Mixed templates containing two or four templates of different subtypes did not interfere with the specificity of dLAMP, and they all detected the H5-subtype template with a concentration of about 300 copies/μL with minor variation, indicating high specificity of the LAMP primers we selected.

Tolerance of dLAMP to Inhibitors. In some cases, the clinical or field-collected samples may contain PCR-inhibitory substances that are difficult to be completely removed, which impairs quantification by qPCR. Here, we tested whether dLAMP will be more resistant to inhibitors and selected two representative substances which are widely present in soil and fecal samples:[31−33] humic acid (HA) and sodium dodecyl sulfate (SDS). For comparison, we simultaneously performed dLAMP, dPCR, and qPCR, using templates of two concentrations (3000 and 100 copies/μL) in the presence or absence of inhibitors. In the absence of inhibitors, qPCR successfully detected the templates with a Ct value of 25 for the template of 3000 copies and 30 for 100 copies. In the presence of either HA or SDS, there was no amplification curve detected by qPCR in any of the three replicates, indicating a complete inhibition of qPCR by either HA or SDS (Figure S3a). dPCR by QX200 was not influenced by 50 ng HA but significantly inhibited by 0.05% SDS (Figure S3b). In comparison, dLAMP succeeded in the amplification of templates regardless of whether inhibitors existed or not and the quantification results were highly consistent in all conditions (Figure 4, panels a and b). Although we did not test all reported inhibitors, these results suggested that dLAMP was very robust and more resistant to inhibition than PCR-based assays. The tolerance ability of dLAMP was likely due to both digital compartmentalization and the use of *Bst* DNA polymerase. Conclusively, dLAMP was more advantageous than dPCR and qPCR in terms of inhibitor tolerance and was potentially more suitable for testing "dirty" samples. Therefore, dLAMP might allow us to incorporate simplified sample preparation for further shortening the delay in diagnosis.

Quantification of Viral Loads from H5N1 Infected Samples. To demonstrate the feasibility of dLAMP to absolute quantification of viral loads in real samples, we tested ten allantoic fluid samples and compared performance of dLAMP with qPCR. These samples were previously verified by reverse-transcription PCR that all of them contained H5N1 viruses (data not shown). As shown in Figure 5, both dLAMP and qPCR succeeded in the detection of viruses from all ten samples and the concentration of each sample measured by both methods were overall close, although certain variation between these two assays occasionally occurred. These results suggested the dLAMP assay performed using the XiE system was quite reliable and could be applied to environmental and clinical samples. Further optimization of the dLAMP efficiency and qPCR standard curve would improve the accuracy of both methods. In this work, we performed 72 h incubation in the allantoic cavity of embryonated hens' eggs for virus propagation and storage. This step is not required for actual samples in clinically relevant assays. In the future, we will evaluate the performance of our dLAMP method in rapid diagnosis using clinical swab samples directly without extra incubation.

■ CONCLUSIONS

In summary, we have established a simple, rapid, and robust dLAMP assay for the absolute quantification of H5-subtype influenza viruses using our newly developed XiE system.[25] Droplet generation, reaction, and detection using XiE occurred in the same well plate without steps of droplet transfer. This integrated strategy helps to effectively simplify operation, minimize droplet loss and coalescence, and speed up readout process. This is important in cases where rapid quantification is required for amount-limited samples. Future work will be directed toward the development of a comprehensive and automated workflow incorporating sample preparation,[34]

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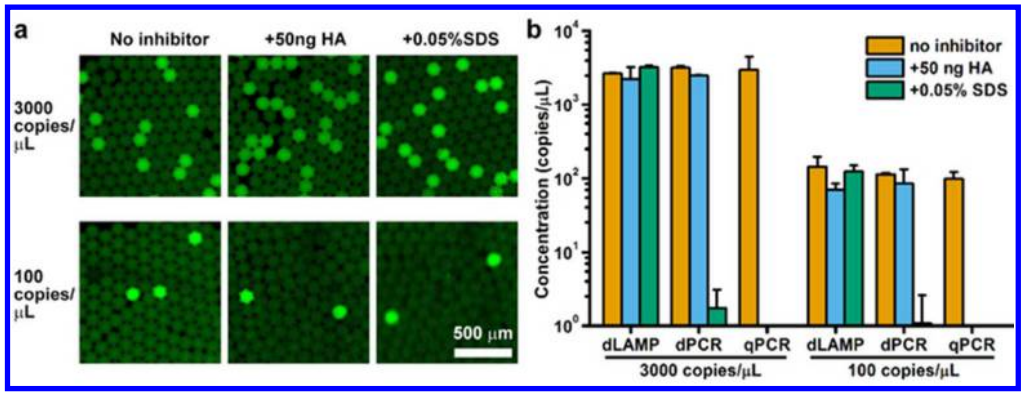

Figure 4. Influence of inhibitors on viral quantification by dLAMP, dPCR, and qPCR. We tested humic acid (HA, 50 ng) and SDS (0.05%) as representative inhibitors and used templates of two concentrations: 3000 and 100 copies/μL. (a) Fluorescence images of dLAMP with and without inhibitors. Scale bar = 500 μm. (b) Quantification of templates by dLAMP, dPCR, and qPCR with and without inhibitors.

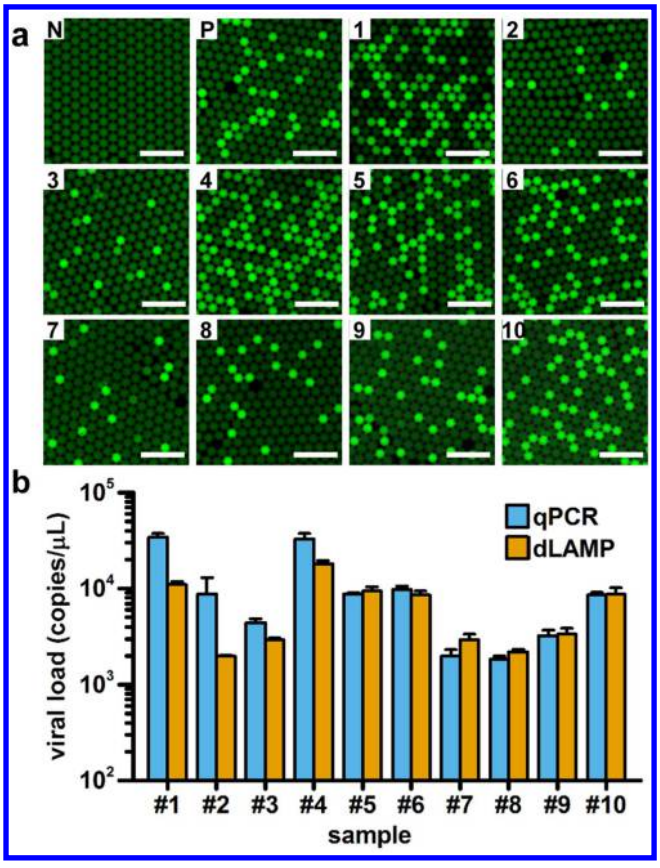

Figure 5. Quantification of H5N1 viral loads in samples harvested from allantoic fluids. (a) Fluorescence images of droplet arrays after the dLAMP reaction for sample nos. 1 to 10 with positive control (P) and negative control (N). Scale bar = 500 μm. (b) Viral load quantification results by qPCR and dLAMP for all samples.

reverse transcription,[35] amplification, and automated fluorescence imaging. We believe that a fully automated XiE platform with a higher throughput and faster droplet generation module will significantly accelerate the use of digital nucleic acids amplification in clinical diagnosis.

In this work, the dLAMP assay showed high reproducibility and sensitivity, which was comparable to current well-acknowledge qPCR and dPCR methods. In addition, the primers used in our dLAMP assay were specifically designed to detect H5 subtype viruses and showed no cross-reactivity to other tested subtypes. Notably, it was highly tolerant to inhibitors that totally inhibited qPCR and dPCR. We also validated that the quantitative analyses by dLAMP were reliable when detecting real H5N1 samples harvested from allantoic fluids. These results indicate that our method can be readily extended to the analysis of human specimens, which will have more clinical significance. We believe that XiE-based dLAMP will have broad applications in the early diagnosis of highly infectious viruses as well as other bacterial and fungal pathogens, enabling dynamic evaluation of treatment and assisting doctors in making timely therapeutic decisions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.6b03328.

Workflow of dLAMP using the XiE method, comparison of the quantification of serial-diluted templates, inhibition assay by qPCR and dPCR (PDF)

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Notes

The authors declare no competing financial interest.

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Absolute Quantification of H5-subtype Avian Influenza Viruses Using Droplet Digital Loop-mediated Isothermal Amplification

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Supporting information

Contents:

SUPPORTING FIGURES:

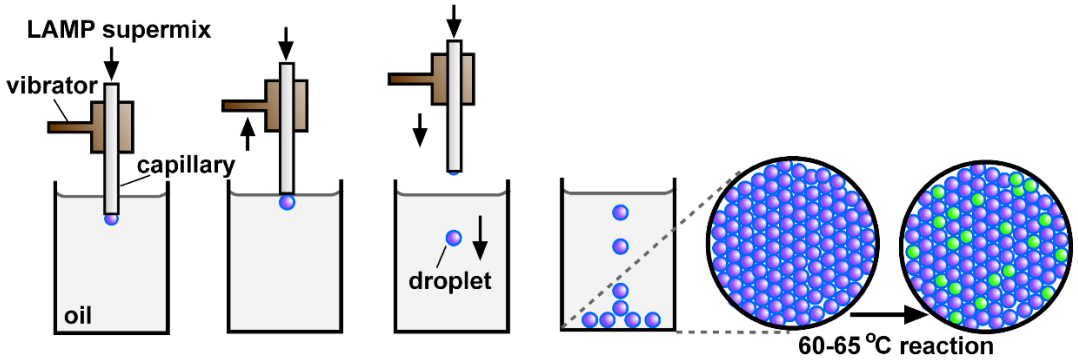

Fig. S1. Workflow of dLAMP usingthe XiE method.

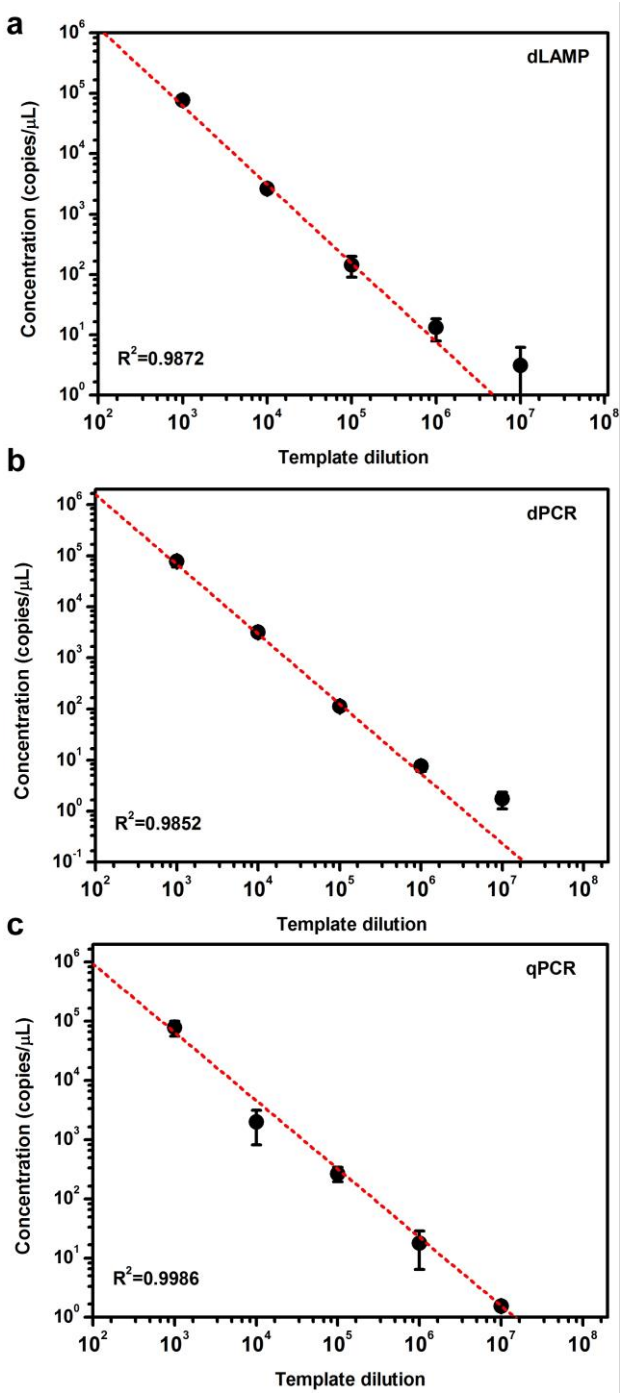

Fig. S2. Comparison of the quantification of serial-diluted templates among dLAMP (a), dPCR (b) and qPCR (c).

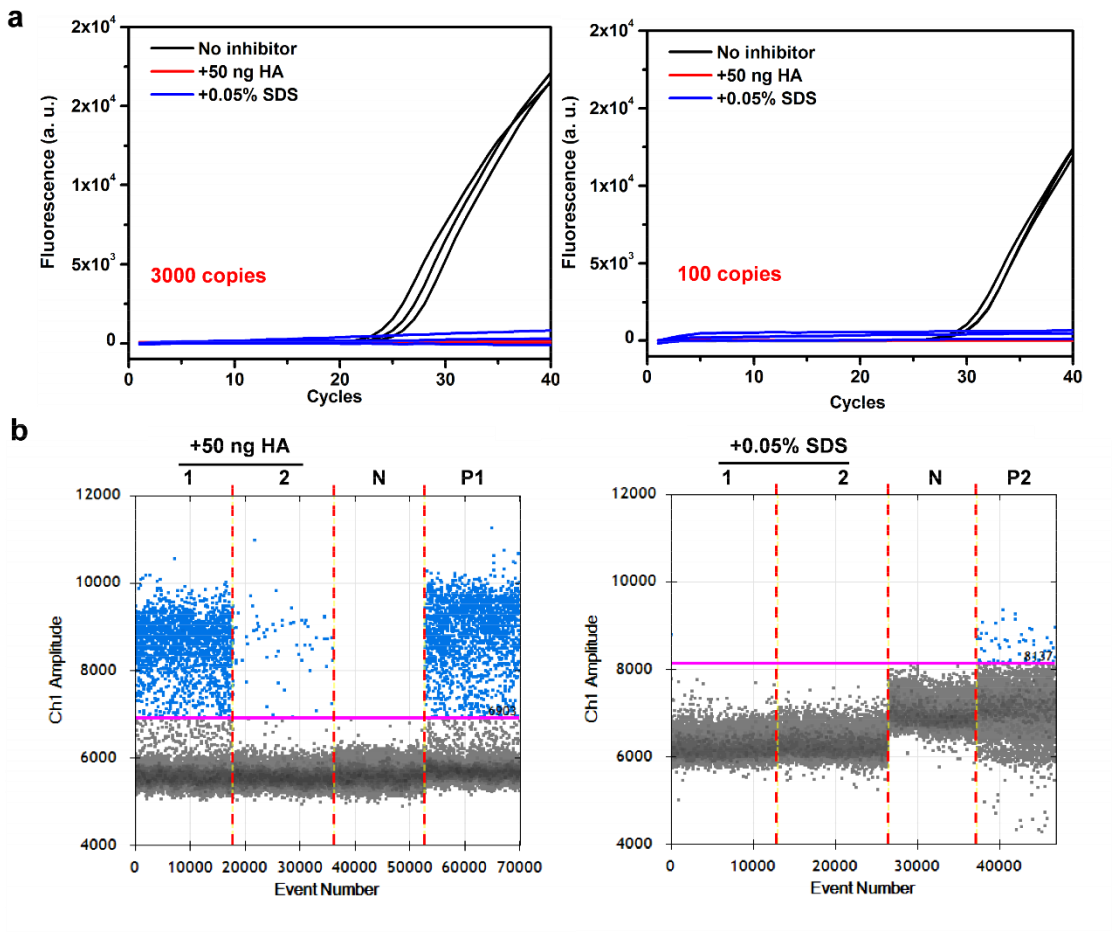

Fig. S3. Inhibition assay by qPCR (a) and dPCR (b). Two inhibitors were introduced into the reaction individually: 50 ng humic acid (HA) and 0.05% SDS. Two amounts of templates were tested: 3000 copies and 100 copies. In (b), 1 and P1=3000 copies; 2 and P2= 100 copies; N=non-template control.