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Letter

Droplet Cas12a Assay Enables DNA Quantification from Unamplified Samples at the Single-Molecule Level

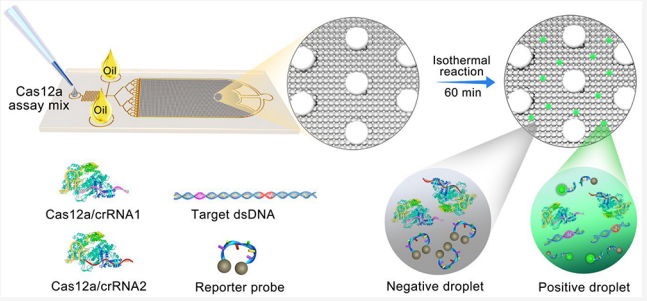
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ABSTRACT: DNA quantification is important for biomedical research, but the routinely used techniques rely on nucleic acid amplification which have inherent issues like cross-contamination risk and quantification bias. Here, we report a CRISPR-Cas12a-based molecular diagnostic technique for amplification-free and absolute quantification of DNA at the single-molecule level. To achieve this, we first screened out the optimal reaction parameters for high-efficient Cas12a assay, yielding over 50-fold improvement in sensitivity compared with the reported Cas12a assays. We further leveraged the microdroplet-enabled confinement effect to perform an ultralocalized droplet Cas12a assay, obtaining excellent specificity and single-molecule sensitivity. Moreover, we demonstrated its versatility and quantification capability by direct counting of diverse virus's DNAs (African swine fever virus, Epstein−Barr virus, and Hepatitis B virus) from clinical serum samples with a wide range of viral titers. Given the flexible programmability of crRNA, we envision this amplification-free technique as a versatile and quantitative platform for molecular diagnosis.



KEYWORDS: *Single-molecule detection, CRISPR-Cas12a, droplet microfluidics, DNA quantification, Molecular diagnosis*

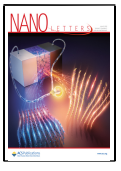
INTRODUCTION

Nucleic acid detection increasingly highlights its importance in the field of biomedical research and clinical applications. For example, as a golden standard for SARS-CoV-2 diagnosis, nucleic acid detection is playing a key role in the control of COVID-19 pandemic outbreak.[1,2] In terms of the application purpose, nucleic acid detection can be divided into qualitative and quantitative ones. Qualitative nucleic acid detection is mainly used for determining the presence of a target of interest, like the diagnosis of pathogen infection.[3,4] Quantitative nucleic acid detection is widely used in those applications where the precise measurement of DNA/RNA levels is crucial, such as antiviral drug screening,[5] methylation analysis,[6] determination of gene mutation frequency,[7] and counting of cell-free DNA for liquid biopsy,[8] etc.

Quantitative PCR (qPCR) is a widely used nucleic acid quantification technology. It quantifies nucleic acids by construction of an external standard curve, which is an indirect quantification method.[9,10] The recently developed digital PCR technology can achieve absolute quantification without the need for external standards.[11−13] However, this technology still requires nucleic acid amplification, inevitably bringing some issues, such as cross-contamination and potential amplification bias.[14] It seems that if nucleic acid detection technology enabling direct quantification of unamplified samples is developed, the above-mentioned issues can be avoided and the simplicity and detection accuracy can be significantly improved. Some existing technologies, such as fluorescence correlation spectroscopy (FCS),[15] coincident fluorescence detection,[16] nanopore,[17] and single-molecule fluorescence resonance energy transfer (FRET),[18] can detect nucleic acids at the single-molecule level. However, these methods depend on molecule diffusion and thus need high target concentration in bulk solution for analysis. This renders them not to be suitable for molecular diagnosis because the concentration of target nucleic acid in biological samples is usually extremely low.

The invention of CRISPR technology provides some exciting opportunities for nucleic acid detection. All single-component protein CRISPR systems discovered so far have proven their merits for developing advanced diagnostic methods.[19−24] In particular, the Cas12 and Cas13 systems, both of which have cis- and trans-cleavage activities, have been widely used in DNA and RNA analysis.[25−28] The trans-cleavage of the Cas12 and Cas13 system, which is specifically

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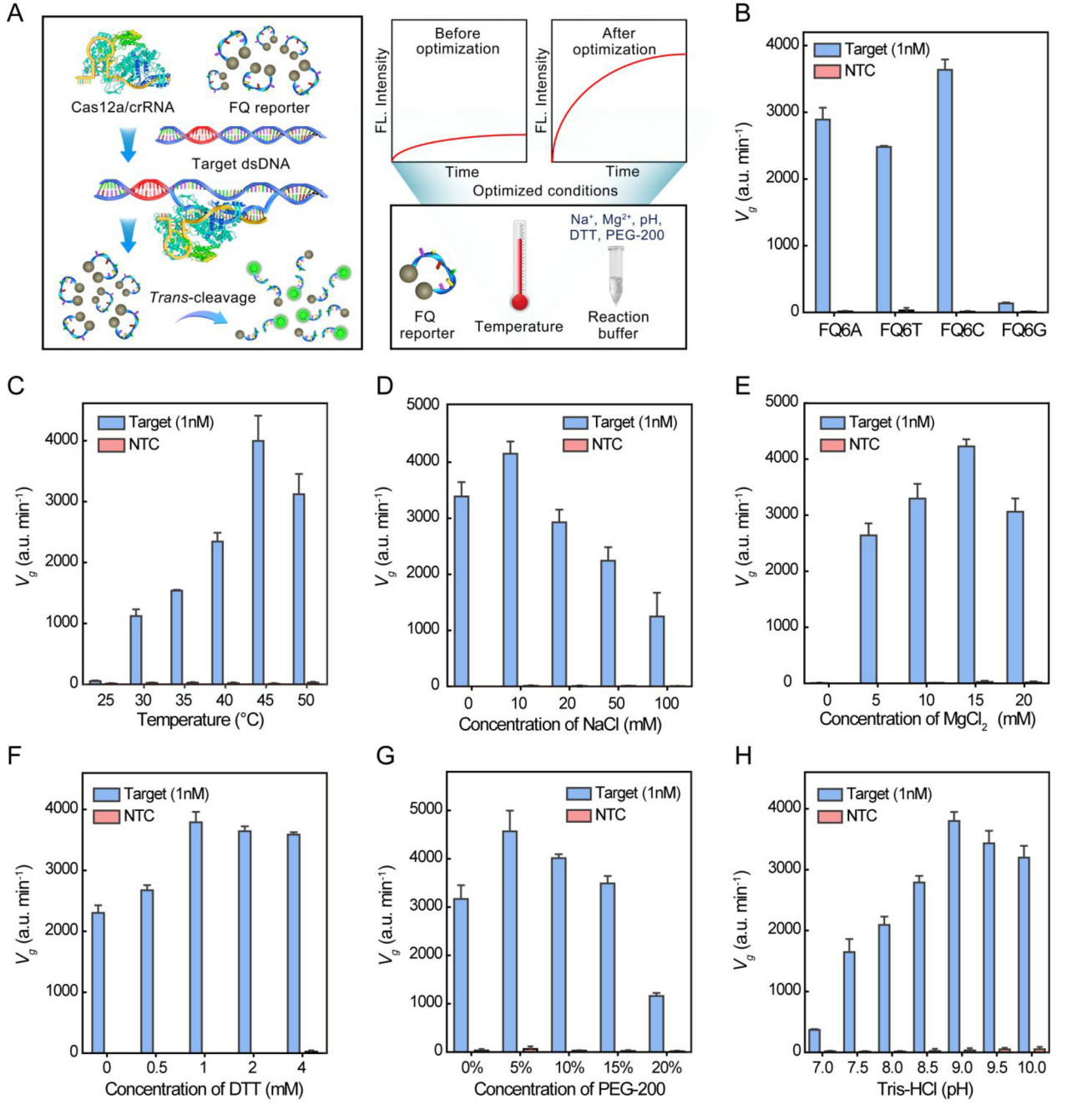


Figure 1. Optimization of the reaction conditions for the Cas12a assay. (A) Cas12a assay is optimized by the homogeneous fluorescence detection of trans-cleavage activity. Reporter sequence, reaction temperature, buffer compositions, and pH are evaluated. Cas12a assay is tested by combination of 100 nM Cas12a and 200 nM crRNA against 1 nM ASFV dsDNA. (B) Effect of reporter probe sequence on Cas12a assay efficiency. (C) Effect of reaction temperature on Cas12a assay efficiency. (D) Effect of Na^+ concentration on Cas12a assay efficiency. (E) Effect of Mg^{2+} concentration on Cas12a assay efficiency. (F) Effect of DTT concentration on Cas12a assay efficiency. (G) Effect of PEG-200 on Cas12a assay efficiency. (H) Effect of buffer pH on Cas12a assay efficiency. All parameters are evaluated by calculating the fluorescence growth rate V_g ($V_g = \Delta F/\Delta t$). Data are represented as mean ± standard error of the difference between means of three technical replicates.

derived from the crRNA-mediated programmed recognition of target DNA/RNA, exhibits a multiple-turnover signal amplification mechanism.[29,30] This trans-cleavage activity is particularly attractive for the development of a new-generation of molecular diagnostic technology, which has put forward higher standards for assay sensitivity, specificity, and convenience.[20,31−33] For example, the LbuCas13a system has an extremely high trans-cleavage activity, exhibiting at least 10^4 turnovers per target RNA recognition.[23] Such high-efficient signal amplification mechanism enables as low as femtomolar RNA can be detected in bulk solution.[34] Particularly, when introducing the LbuCas13a assay to picoliter-scale droplets, we have demonstrated a >10 000-fold enhancement in sensitivity when compared to the bulk Cas13a assay, thus enabling universal and amplification-free single-molecule RNA quantification.[35] This unprecedented sensitivity enhancement based on an ultralocalized concept motivated us to develop an amplification-free DNA quantification technology at single-molecule level using CRISPR-Cas12a system. However, to the

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Nano Lett. 2021, 21, 4643−4653

A

	Component	References
Buffer 1	20 mM HEPES (pH 7.5), 150 mM KCl, 10 mM $MgCl_2$, 1% glycerin,0.5 mM DTT	20
Buffer 2	10mM Tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, 100 μg/mL BSA	32
Buffer 3	20 mM HEPES (pH 6.8), 60 mM NaCl, 6 mM $MgCl_2$	31
Buffer 4	10mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 100 μg/mL BSA	39
Enhanced Buffer	10mM Tris-HCl (pH 9.0), 10 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT, 5% PEG-200	This study

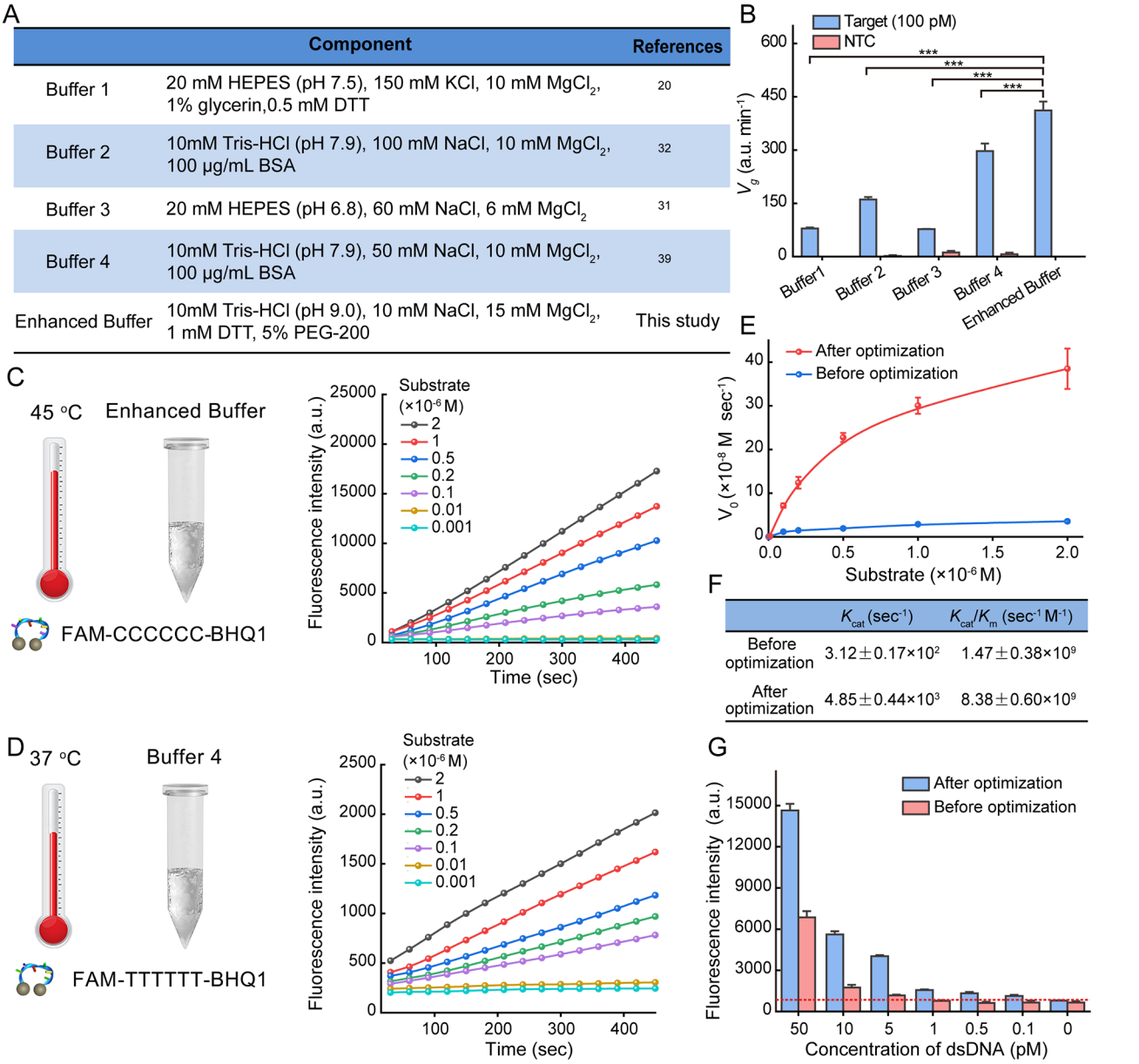


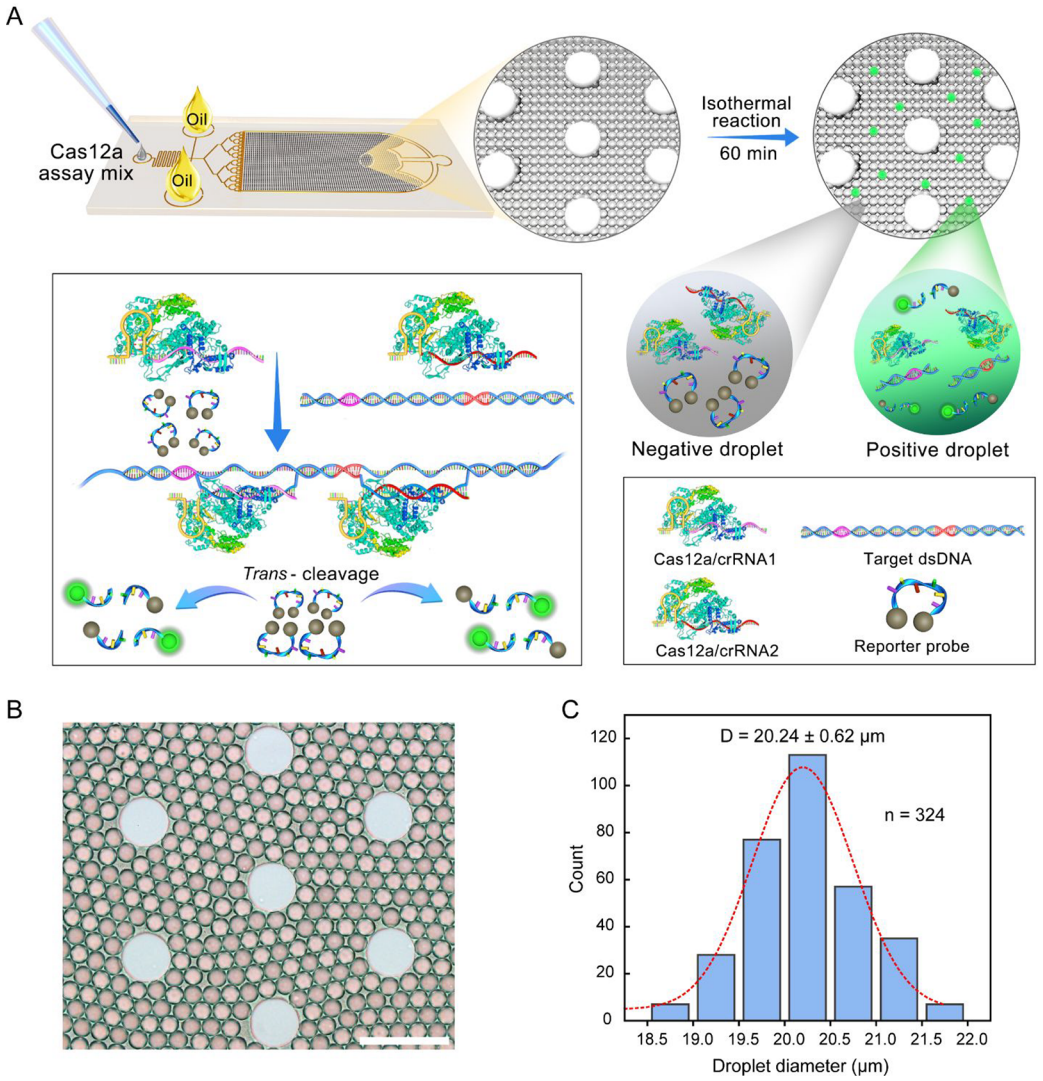
Figure 2. Evaluation of the optimized Cas12a assay. (A) Compositions of the obtained enhanced buffer and other three kinds of typical Cas12a assay buffers. (B) Comparison of Cas12a assay efficiency using different buffers. Cas12a assay is performed by using the combination of 100 nM Cas12a and 200 nM crRNA against 100 pM ASFV dsDNA. The efficiency of Cas12a assay is evaluated by calculating the fluorescence growth rate. Data are represented as mean ± standard error of the difference between means of three technical replicates. The differences between two groups were analyzed by two-sided Student *t*-tests ($p < 0.0001$). Cas12a assay efficiency is evaluated by calculating the fluorescence growth rate V_g. Data are represented as mean ± standard error of the difference between means of three technical replicates. (C−D) Representative plots of initial velocity versus time under the optimal reaction parameters (enhanced buffer, 45 °C reaction, and C6 reporter) and the conventional reaction parameters (buffer 4, 37 °C reaction and T6 reporter). Here, 0.1 nM Cas12a-crRNA-activator complex was used. (E) Michaelis−Menten kinetic study for the corresponding optimal (C) or conventional (D) reaction parameters. Data are represented as mean ± standard error of the difference between means of three technical replicates. (F) Enzyme kinetic data of Cas12a before and after optimization. (G) Comparison of the sensitivity performance of the Cas12a assay before and after optimization. Data are represented as mean ± standard error of the difference between means of three technical replicates. The red dotted line represents the detection limit, which was determined as mean of NTC plus standard error ×3.

best of our knowledge such a technique has not yet been reported so far.

Compared with the Cas13a system, the reported trans-cleavage efficiency of Cas12a is about 1−2 order of magnitude lower. Such efficiency only allows the direct detection of DNA molecules at picomolar range, which renders the droplet-based single-molecule DNA detection without nucleic acids amplification to be a huge challenge.[36] In this study, we have comprehensively investigated the effect of reaction conditions on Cas12a reaction efficiency. As a result, we found that the Cas12a reaction efficiency can be significantly improved by optimizing the buffer, reaction temperature, and trans-cleavage substrate. Using the optimized reaction conditions, our high-efficient Cas12a assay can directly detect DNA at the 100 fM level in bulk solution, exhibiting a 50-fold improvement when compared to the existing Cas12a assays.[20,37] Further, by developing a dual-crRNA targeting droplet assay strategy, our highly efficient droplet Cas12a assay could achieve single-

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Nano Lett. 2021, 21, 4643−4653

Scheme 1. Development of Dual-crRNA Targeting Droplet Cas12a Assay[a]



[a](A) A microfluidic droplet chip is designed with a sample inlet and a pair of oil inlets for generating a highly uniform monolayer droplet array by negative pressure. Cas12a assay mix contains Cas12a protein, two crRNAs, reporter probe, and with and without target DNA. Subsequently, the formed droplets undergo an isothermal reaction for 60 min. At low target DNA concentration, the vast majority of droplets would contain 0 or 1 target DNA and can be *in situ* detected by fluorescence imaging-based droplet counting. (B) Highly uniform microdroplets were generated for current droplet Cas12a assay. Imaging is executed under 20× objective lens (scale bar: 100 μm). (C) Size distribution of the droplets.

molecule digital DNA quantification. We successfully demonstrated its utility for specific and quantitative detection of African swine fever virus (ASFV), Epstein−Barr virus (EBV), and Hepatitis B virus (HBV) from clinical serum samples without the need of nucleic acid amplification. We anticipate that the developed droplet Cas12a assay could potentially serve as a next-generation DNA quantification technique by harnessing the flexible programmability of crRNA.

RESULTS

Reinvestigation of the Reaction Conditions for CRISPR-Cas12a Assay. Although CRISPR-Cas12a and CRISPR-Cas13a have similar trans-cleavage mechanism, the sensitivity of the Cas12a assay is 1−2 orders of magnitude lower than that of the Cas13a assay. In this study, we seek to improve the efficiency of LbCas12a assay by optimizing the reaction parameters, such as buffer conditions, reaction temperature and trans-cleavage substrate. Fluorescent Cas12a assay experiments are executed by targeting a DNA sequence derived from ASFV (Figure 1A). To evaluate these effects quantitatively, trans-cleavage activity of Cas12a assay was represented by fluorescence growth rate V_g ($V_g = \Delta F/\Delta t$).

Initially, we investigated the effect of the reporter sequence on trans-cleavage efficiency. Subconsciously, a short T-rich DNA FRET probe, derived from the first two reports of discovering trans-cleavage mechanism, is conventionally adopted.[20,21] However, a very recent report showed that a C-rich substrate is more efficient[38] whereas another published work achieved maximal trans-cleavage activity with TA-rich substrate.[39] Here, we screened reporter probes with base composition of 6A, 6T, 6C, and 6G, to evaluate their efficiency, respectively. It is showed that 6C is the most suitable substrate for Cas12a assay system (Figure 1B, Figure S1A). Further extension of the number of C bases impaired the detection signal and the background signal also increased (Figure S1B,C).

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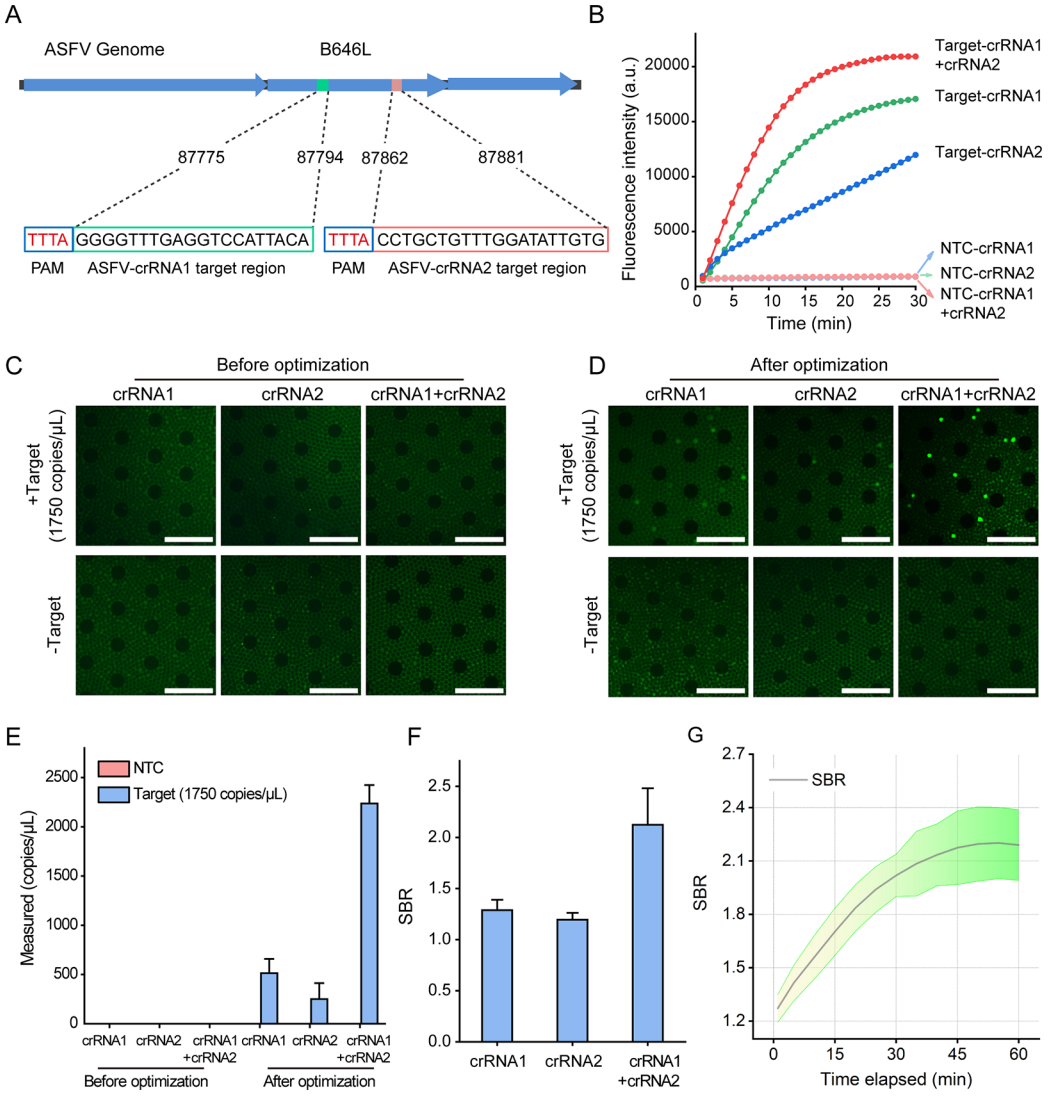


Figure 3. Verification of the single-molecule detection ability of the droplet Cas12a assay. (A) Schematic of the ASFV genome and corresponding location of spacer region of two B646L gene targeted crRNAs. (B) Cas12a assay made with ASFV-crRNA1 and ASFV-crRNA2 individually and in combination were executed against 100 pM ASFV DNA. NTC represents no target DNA control. (C,D) Representative end-point fluorescence images of droplet Cas12a assay using ASFV-crRNA1 and ASFV-crRNA2 individually or in combination under unoptimized conditions (buffer 4, 37 °C, and 6T reporter) and optimized conditions (enhanced buffer, 45 °C reaction, and 6C reporter). Here, 1.75×10^3 copies/μL ASFV DNA or no target DNA control were used (10× magnification, scale bar: 200 μm). (E) Counting of the DNA copy numbers using individual or combined crRNA sets under unoptimized and optimized droplet Cas12a assays. (F) Signal-to-background ratio (SBR) of single crRNA or dual-crRNA at 60 min. (G) Time course showing the SBR of the fluorescence intensity of the positive droplet to the negative droplet (mean ± SD, n = 5).

We next evaluated the temperature dependence of the Cas12a assay. The Cas12 family, including Cas12a, Cas12b, and Cas12f, has unique reaction temperature characteristics. For example, Cas12b and Cas12f are generally considered to be more efficient at 40−50 °C,[40,41] while Cas12a is routinely employed for testing at 37 °C.[32,37] In this study, we varied the reaction temperature from 25 to 50 °C with an interval of 5 °C and found the reaction temperature has a significant effect on LbCas12a assay efficiency. The best signal quality was obtained at 45 °C. It seems that high reaction temperature not only increases the reaction rate but also enhances the total trans-cleavage activity for LbCas12a (Figure 1C, Figure S1D).

We further evaluated the efficiency of CRISPR-Cas12a assay by finely adjusting the composition and concentration of the reaction buffer. We found that a buffer composing of 10 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT, and 5% PEG-200 is beneficial to improve the efficiency of Cas12a assay (Figure 1D−G, Figure S1E−H). Importantly, unlike most buffers that use neutral pH, our experiments indicated that Cas12a assay has a significant efficiency improvement under weak alkaline conditions (pH 9) (Figure 1H, Figure S1I). A recent study indicated that substituting Mn^{2+} for Mg^{2+} get better *trans*-cleavage efficiency,[42] but our research result showed that Mg^{2+} is the most efficient (Figure S2A,B). Other divalent cations, such as Fe^{2+}, Cu^{2+}, and Ca^{2+} were also evaluated, but none of them exhibited a beneficial promotion (Figure S2C,D).

Evaluation of the Optimized Cas12a Assay. Next, we investigated the performance of the optimized Cas12a buffer system by comparing the outcome with those of other widely adopted buffers for Cas12a (Figure 2A). These buffers include Buffer 1, a buffer used in DETECTOR technology;[20] Buffer 2, a buffer used to build HOLMES platform;[32] Buffer 3, a buffer used in SHERLOCK assay;[31] and Buffer 4, also known as NEBuffer 2.1,[39] a buffer from the commercial Cas12a kit. As

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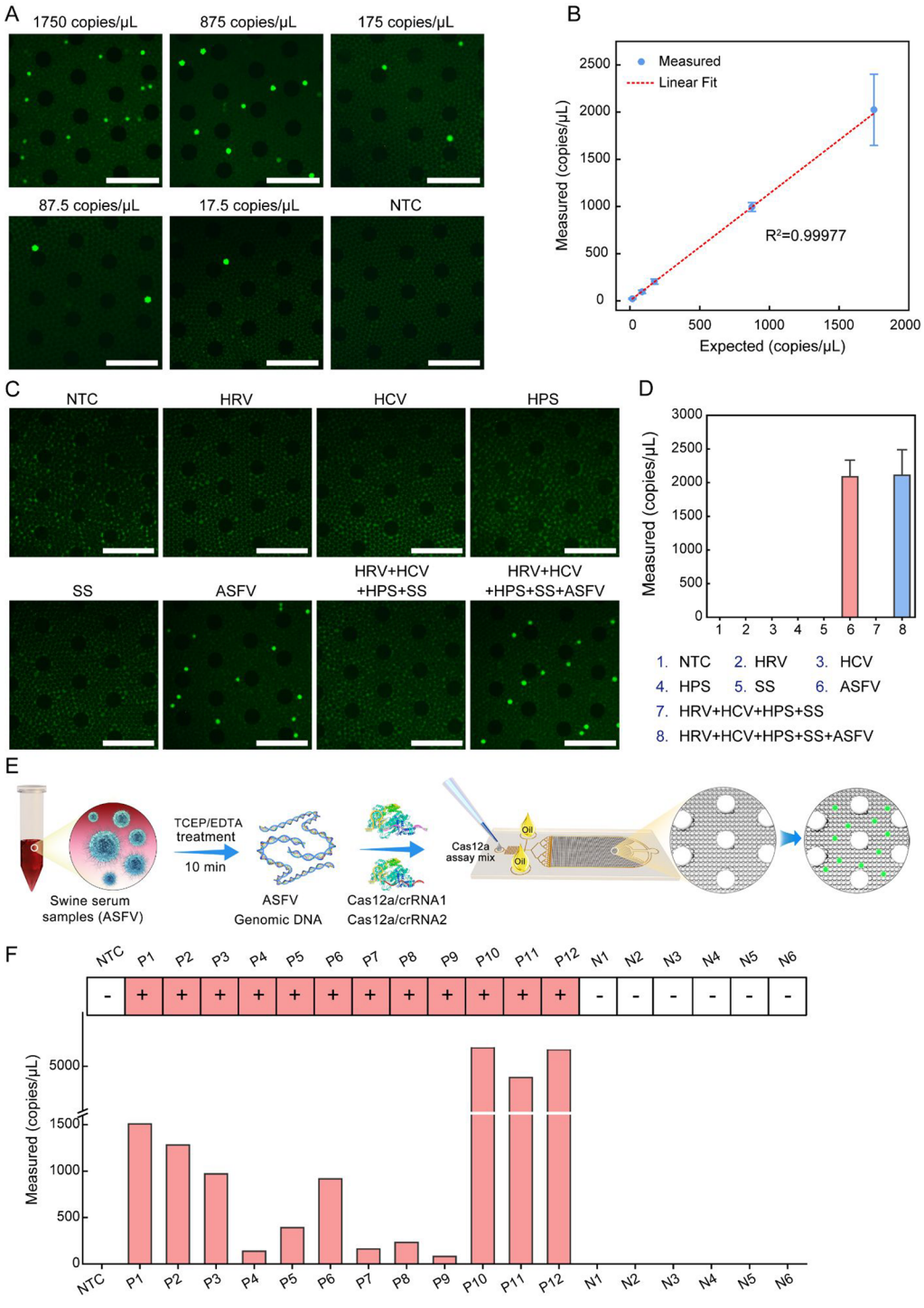


Figure 4. Verification of the sensitivity, quantifiability, specificity, and applicability of the droplet Cas12a assay. (A) Representative end-point fluorescence images of dual-crRNA targeting droplet Cas12a assay for analysis of 1750, 875, 175, 87.5, and 17.5 copies/μL of ASFV genome DNA (10× magnification, scale bar: 200 μm). NTC represents no target DNA control. (B) Linear response analysis for quantification range of the droplet Cas12a assay. Data are represented as mean ± standard error of the difference between means of three technical replicates. (C) Representative end-point fluorescence images of droplet Cas12a assay for analyzing *Pseudorabies virus* (PRV), *Porcine circovirus* (PCV), *Streptococcus suis* (SS), *Haemophilus parasuis* (HPS), and ASFV genome DNA, using ASFV specific dual crRNAs. (D) Counting of the DNA copy numbers in PRV, PCV, HPS, SS, and ASFV genomic DNA by ASFV-targeted dual-crRNA droplet Cas12a assay. (E) Schematic of ASFV detection in suspected swine serum samples by dual-crRNA targeting droplet Cas12a assay. (F) Quantitative measurement of viral load in 18 ASFV-suspected swine serum samples. These swine serum samples were also confirmed by qPCR. NTC represents no target DNA control. P1−P12 represents positive samples. N1−N6 represents negative samples.

shown in Figure 2B, our enhanced buffer yielded a significantly faster enzymatic reaction speed when compared with buffer 1 to 3, and a better efficiency than that of the commercial NEBuffer 2.1 (Figure 2B, Figure S3). To further investigate the enzymatic activity of Cas12a under the optimal reaction parameters, we performed a Michaelis−Menten kinetic analysis

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Nano Lett. 2021, 21, 4643−4653

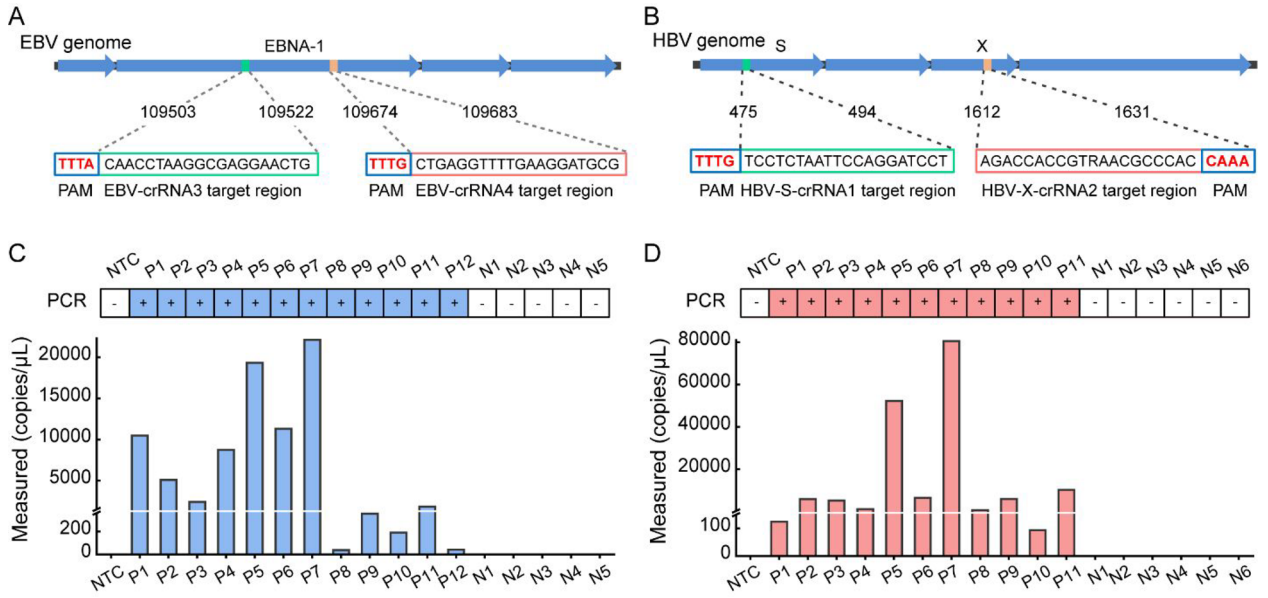


Figure 5. Droplet Cas12a assay for human-associated viruses. (A) Schematic of the EBV genome and corresponding location of spacer region of two EBNA-1 gene targeted crRNAs. (B) Schematic of the HBV genome and corresponding location of spacer region of S gene and X gene targeted crRNAs. (C) Quantitative measurement of viral load in 17 EBV-suspected serum samples. These serum samples were also confirmed by qPCR method. NTC represents no target DNA control. P1−P12 represents positive samples. N1−N5 represents negative samples. (D) Quantitative measurement of viral load in 17 HBV-suspected serum samples. These serum samples were also confirmed by qPCR method. NTC represents no target DNA control. P1−P11 represents positive samples. N1−N6 represents negative samples.

under the optimal and preoptimal reaction parameters (Figure 2C,D). The result showed that a 5.7-fold improvement of K_{cat}/K_m ratio was obtained after optimization (Figure 2E,F). Next, we evaluated the sensitivity of the CRISPR-Cas12a assay under the optimal reaction parameters. By using serially diluted ASFV DNA samples, we observed that as low as 100 fM DNA can be detected with easily distinguishable signals above the background (Figure S4A,B). It signified that the current Cas12a assay is 50-fold more sensitive than that of the conventional Cas12a assay (Figure 2G, Figure S4C,D). Interestingly, we found that the efficiency of cis-cleavage in the optimized buffer was also significantly improved (Figure S5). In the optimized buffer, 10 nM target DNA could be utilized completely by 100 nM Cas12a within 20 min, whereas it took about 30 min to achieve a comparable outcome in the NEBuffer 2.1.

Development of Dual-crRNA Targeted Droplet Cas12a Assay. After establishing a high-efficient CRISPR-Cas12a assay, we envisaged the possibility for achieving single-molecule DNA quantification by confining this assay in a microdroplet. This strategy has endowed the Cas13a assay with the single-molecule RNA quantification capability.[35] To achieve this, we made two important modifications tailored for the current droplet Cas12a assay (Scheme 1A). First, we reconfigured the microfluidic chip to generate smaller droplets for further enhancement of the local concentration of both target DNA and signal reporters. The redesigned chip system can produce droplets with a diameter of 20 μm (Scheme 1B,C), which has over 3.4-fold volume reduction compared with the droplet used in our previous version.[35] Second, we leveraged a dual-crRNA targeting strategy to further improve Cas12a assay efficiency, as it has been proven to be powerful in a Cas13a-based SARS-CoV-2 detection assay.[34] Here, four crRNA candidates for targeting B646L gene were evaluated and two crRNAs (ASFV-crRNA1 and ASFV-crRNA2) showed better detection efficiency (Figure S6). As expected, bulk ASFV DNA detection with c ASFV-crRNA1 and ASFV-crRNA2 dual-targeted strategy obtained more robust trans-cleavage efficiency than the single crRNA test (Figure 3A,B, Figure S7).

To check the single-molecule DNA detection capability, we used 10 fM ASFV DNA as the target to perform droplet Cas12a assay. At such a DNA concentration, each droplet contains zero or one DNA molecule with a probability of >99%. In order to determine whether the optimization of the Cas12a assay is critical for the droplet-based detection, comparison tests were also executed using unoptimized conditions. The results showed that neither single crRNA nor dual-crRNA targeting droplet Cas12a assay could effectively yield expected positive droplets using unoptimized conditions (Figure 3C). However, positive droplets with weak fluorescence can be observed in single-crRNA targeting Cas12a assay under optimized conditions (Figure 3D). In contrast, the dual-crRNA targeting Cas12a assay exhibited improved positive droplet counts (Figure 3E) and brightness (Figure 3F) without causing undesirable background interference. A signal-to-background ratio (SBR) > 2 is obtained with 45 min (Figure 3G). A comprehensive statistic shows that the measured DNA concentration is about 2.24×10^3 copies/μL, which is close to the input concentration (1.75×10^3 copies/μL) measured by digital PCR (Figure 3E). This slight difference might result from the measuring deviation between the two analytical techniques.

Demonstration of the Sensitivity, Quantifiability, Specificity, and Applicability of the Droplet Cas12a Assay. Having successfully demonstrated the single-molecule detection capability of droplet Cas12a assay, we further evaluated that whether the droplet Cas12a assay can be quantitative and specific. Here, a known concentration of ASFV genomic DNA quantified by digital PCR is used as the target for testing the quantitative ability. Six serially diluted

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DNA samples with the concentrations of 1750, 875, 175, 87.5, 17.5, and 1.75 copies/μL, were subjected to the droplet Cas12a assay. Results showed that the measured positive droplets are precisely correlated to the input DNA concentration from 1750 to 17.5 copies/μL with an excellent linear response ($R^2 > 0.999$) (Figure 4A,B).

The input sample used in the above experiments was the purified ASFV genomic DNA. This led us to a concern about whether the detection signal is specific when complex samples are used. We then used six kinds of bacterial genomic DNAs, including *Listeria monocytogenes* (Lm), *Staphylococcus aureus* (Sa), *Neisseria encephalitis* (Ne), *Salmonella typhimurium* (St), *Enterobacter sakazakii* (Es), *and Pseudomonas aeruginosa* (Pa) as the interference samples to challenge the detection specificity. These bacterial DNA sequences are larger than 1 million bases, which should give enough sequence diversity for specificity analysis. Results showed that no positive droplets were observed in the control experiments and all six bacterial genomic DNA groups (Figure S8A). We next challenged its specificity by detecting the pure ASFV DNA samples and ASFV DNA mixed with all six kinds of bacterial DNAs, respectively. Results showed that almost identical positive droplet proportions were obtained in these two experiments, which fully proved the detection fidelity (Figure S8A,B). We further used the genomic DNA extracted from four pathogenic bacteria (*Pseudorabies virus* (PRV), *Porcine circovirus* (PCV), *Haemophilus parasuis* (HPS), and *Streptococcus suis* (SS)) that cause swine respiratory diseases as interference to evaluate the specificity of the developed method. Neither a single pathogenic DNA nor multiple pathogenic DNA mixtures cause any interference signals. Meanwhile, ASFV crRNA-targeted droplet Cas12a assay also shows excellent specificity for selectively counting ASFV DNA from these pathogenic DNA mixtures (Figure 4C,D).

To challenge its applicability for direct detection of unamplified DNAs in real-world samples, we performed droplet Cas12a assay to detect 18 ASFV-suspected swine serum samples (Figure 4E). These DNA materials in the serum samples were released by using a rapid and simple TCEP/EDTA thermal reduction method[43] and then confirmed by qPCR. We then directly analyzed these released DNAs with dual-crRNA targeting droplet Cas12a assay. Encouragingly, this assay detected all positive and negative samples correctly, with the results completely consistent with those of the qPCR (Figure S9, Table S3). Moreover, the droplet quantification results showed that the viral DNA concentration of these positive samples ranges from approximately 80 to 5200 copies/μL, while their corresponding Ct values range from 20.88 to 34.52 (Figure 4F and Table S3).

Droplet Cas12a Assay for Detection of Human-Associated Viruses in Clinical Serum Samples. To further verify its applicability to other real-word samples, we applied this assay to detect two human-associated viruses in clinical serum samples (Figure 5). Here, Epstein−Barr virus (EBV) and Hepatitis B virus (HBV) were chosen as models for the following reasons. EBV DNA is a useful clinical and research biomarker for nasopharyngeal carcinoma (NPC), and the quantitative detection of EBV DNA in serum samples represents a powerful liquid biopsy tool to screen for early NPC in asymptomatic persons.[44,45] HBV infection is a global public health threat due to the considerable liver-related morbidity and mortality; the serum HBV DNA concentration is closely related to disease progression and the efficacy of drug treatment.[46,47]

To accommodate these quantification applications, we first executed the corresponding crRNAs' design and screening (Figure 5A,B). Given that EBNA-1 gene, HBV S, and X gene are widely used for clinical detection,[47,48] the corresponding crRNAs were designed to target these genes. EBV-crRNA3/-crRNA4 and HBV-S-crRNA1/-X-crRNA2 were chosen for dual-crRNA targeted droplet Cas12a assay because of the relatively high efficiency (Figures S10 and S11). The clinical samples chosen for this testing were first approved by qPCR with measured Ct values ranging from 20.82 to 36.99 (Figures S12 and S13 and Table S3). Then, we tested them using the sample protocol and cocktail described in ASFV DNA detection, other than crRNA pairs. We found that droplet Cas12a assay correctly identified all positive and negative samples of EBV and HBV with 100% concordance with qPCR results (Figure 5C,D). Importantly, the measured DNA concentrations for all positive samples ranged from 37 to 80 553 copies/μL, which are correctly relative to their measured Ct values.

DISCUSSION

Recently, we have developed a single-molecule RNA quantification technology via microdroplet-powered Cas13a assay.[35] Encouraged by this successful strategy, here we seek to develop single-molecule DNA quantification technology using the Cas12a system. However, our preliminary experiments suggested that the ordinary droplet Cas12a assay has not yet achieved single-molecule DNA detection even when the most efficient LbCas12a enzyme is adopted. Notably, several reports also indicated that the direct bulk LbCas12a assay could only detect 10 pM of target DNAs,[36,49] while LbuCas13a assay can achieve fM-level sensitivity for RNA.[34] Here, we turned to a systematically optimization of the reaction conditions for improving Cas12a assay efficiency. Our screening experiments showed that Cas12a assay efficiency could be significantly improved when combining the enhanced buffer (10 mM Tris-HCl (pH 9.0), 10 mM NaCl, 15 mM $MgCl_2$, 1 mM DTT, 5% PEG-200), 6C reporter and 45 °C reaction temperature. Under these optimized parameters, DNA targets as low as 100 fM could be detected. This means that significant enhancement in Cas12a reaction efficiency is obtained when compared with the conventional Cas12a assay.

Such a significant efficiency enhancement resparked a great hope in developing a droplet Cas12a assay for single-molecule DNA detection. Furthermore, incorporating with dual-crRNA targeting strategy, the high-efficient droplet Cas12a assay indeed provides single-molecule DNA quantification. We experimentally proved that the combinational optimization for droplet Cas12a assay allows as low as 17.5 copies/μL ASFV DNA to be reliably quantified. Since only about 1 μL of sample is consumed, it means that several copies of DNA can be counted. It is worth noting that we have also challenged the sensitivity of the droplet Cas12a assay by further diluting the sample to 1.75 copies/μL. However, we only successfully detected the expected DNA molecule in one of three independent experiments (data not shown). We suspect that the low repeatability at such an extremely low concentration may be attributed to the adsorption effect. Despite this, we have experimentally proven that the droplet Cas12a assay could quantify unamplified DNAs at the single-molecule level. More importantly, droplet Cas12a assay omits the DNA

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Nano Lett. 2021, 21, 4643−4653

replication process, thus avoiding cross-contamination risk. This will be a conceptual advance because it means that the construction of molecular diagnostic laboratories using this method will be simpler and cheaper. Conventional construction of PCR-based molecular diagnostic laboratories requires physical space separation for sample processing, amplification, and detection steps, as well as a strict air conditioning system to avoid cross-contamination, and thus is extremely expensive.

The second key advance of the droplet Cas12a assay is its inherent quantification ability. Quantitative DNA detection is very important in basic life science research and medical diagnosis.[5−8] The droplet Cas12a assay could count DNA molecules by digital droplet analysis, thus providing absolute quantification of DNA. An excellent (R^2 > 0.999) linear response between the input and measured DNA concentration was observed, which suggests that this assay is capable of precise DNA quantification. Compared with the widely used qPCR,[9,10] droplet Cas12a assay is advantageous in direct and absolute DNA quantification without the need for external standards. Compared with the state-of-art digital PCR technology,[11−13] droplet Cas12a assay enables the direct counting of single unamplified DNA molecules, thus avoiding potential amplification errors caused by enzyme and primers, so it could be more accurate in principle. Compared with the recently developed nucleic acids amplification-based digital Cas12a-based strategies,[36,50,51] current droplet Cas12a assay quantitatively detects DNA from unamplified samples, so it is more accurate, simpler, and can prevent undesired cross-contamination issues.

Notably, we explored its applicability for precise counting of ASFV DNAs, specific discrimination of ASFV DNAs from six bacterial genomic DNAs and five porcine respiratory pathogen DNAs, and direct quantification of ASFV DNAs from TCEP/EDTA thermal reduction treated serum samples. Moreover, the performance in detection of the human-associated viruses further confirmed its applicability for precise quantification of real-world samples with a wide range of viral titers. These results indicated that the optimized reaction parameters (including temperature, trans-cleavage substrate, buffer component) also accommodate other species-specific DNA detection, by simply replacing the crRNA sequence pairs. Thus, the sensitive, specific, and universal droplet Cas12a assay has robust scalability and practicability in the field of molecular diagnosis.

CONCLUSIONS

In conclusion, we have demonstrated the concept and feasibility of a single-molecule DNA quantification technique via droplet Cas12a assay. To the best of our knowledge, this is the first reported DNA detection method that enables absolute quantification of unamplified DNAs at the single-molecule level. Because of the exhibited sensitivity, specificity, and quantifiability, we anticipate this method can be widely adopted in quantitative biomedical research and applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.1c00715.

Details of materials and methods; optimization of the CRISPR-Cas12a detection system (Figure S1); evaluation of the divalent cation effect on Cas12a assay efficiency (Figure S2); real-time fluorescent kinetic measurements of the trans-cleavage activity of Cas12a with various buffers (Figure S3); evaluation of the sensitivity of the Cas12a assay before and after optimization (Figure S4); comparison of the cis-cleavage activity of Cas12a assay in NEBuffer 2.1 and enhanced buffer (Figure S5); real-time fluorescent kinetic measurements of the trans-cleavage activity of Cas12a with ASFV-crRNA1, ASFV-crRNA2, ASFV-crRNA3, and ASFV-crRNA4 for targeting B646L gene of ASFV (Figure S6); real-time fluorescent kinetic measurements of Cas12a assay with dual-crRNA targeted strategy (Figure S7); verification of the specificity of the droplet Cas12a assay (Figure S8); qPCR detection of 12 clinical ASFV-infected positive swine serum samples and 6 negative samples (Figure S9); real-time fluorescent kinetic measurements of the trans-cleavage activity of Cas12a with EBV-crRNA1, EBV-crRNA2, EBV-crRNA3, and EBV-crRNA4 for targeting EBNA-1 gene of EBV (Figure S10); real-time fluorescent kinetic measurements of the trans-cleavage activity of Cas12a with HBV-S-crRNA1 and HBV-S-crRNA2 for targeting S gene, and HBV-X-crRNA1 and HBV-X-crRNA2 for targeting X gene of HBV (Figure S11); qPCR detection of 12 clinical EBV-infected positive samples and 5 negative samples (Figure S12); qPCR detection of 11 clinical HBV-infected positive samples and 6 negative samples (Figure S13); sequences used in this work (Table S1); buffers used in this work (Table S2); Ct values of qPCR for viral DNA detection from real-world samples (Table S3) (PDF)

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H.H.Y., B.W.S., and X.M.Z. designed the research, H.H.Y. and B.W.S performed the research, D.B.Z., J.S., Q.L., S.C.W., and Y.R.L. contributed new reagents, T.T., E.H.X., and M.Q.H. analyzed the data, H.H.Y. and X.M.Z. wrote the paper, and B.W.S. edited the paper.

Notes

The authors declare no competing financial interest.

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Nano Lett. 2021, 21, 4643−4653