



Universally Stable and Precise CRISPR-LAMP Detection Platform for Precise Multiple Respiratory Tract Virus Diagnosis Including Mutant SARS-CoV-2 Spike N501Y

Tong Zhang, Wei Zhao, Wang Zhao, Yuying Si, Nianzhen Chen, Xi Chen, Xinlian Zhang, Lieying Fan,* and Guodong Sui*



Cite This: *Anal. Chem.* 2021, 93, 16184–16193



Read Online

ACCESS |



Metrics & More

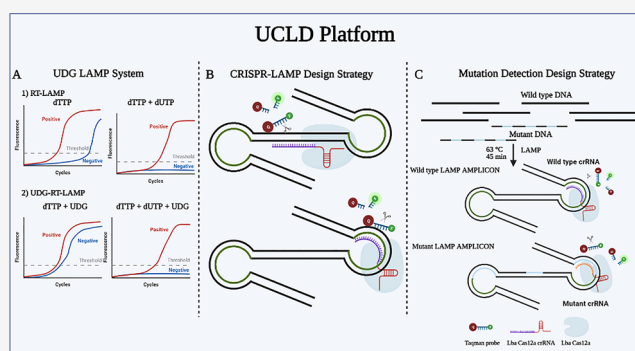


Article Recommendations



Supporting Information

ABSTRACT: Nowadays, rapid and accurate diagnosis of respiratory tract viruses is an urgent need to prevent another epidemic outbreak. To overcome this problem, we have developed a clustered, regularly interspaced short palindromic repeats (CRISPR) loop mediated amplification (LAMP) technology to detect influenza A virus, influenza B virus, respiratory syncytial A virus, respiratory syncytial B virus, and severe acute respiratory syndrome coronavirus 2, including variants of concern (B.1.1.7), which utilized CRISPR-associated protein 12a (Cas12a) to advance LAMP technology with the sensitivity increased 10 times. To reduce aerosol contamination in CRISPR-LAMP technology, an uracil-DNA-glycosylase-reverse transcription-LAMP system was also developed which can effectively remove dUTP-incorporated LAMP amplicons. In vitro Cas12a cleavage reaction with 28 crRNAs showed that there were no position constraints for Cas12a/CRISPR RNA (crRNA) recognition and cleavage in LAMP amplicons, and even the looped position of LAMP amplicons could be effectively recognized and cleaved. Wild-type or spike N501Y can be detected with a limit of detection of 10 copies/ μL (wild-type) even at a 1% ratio level on the background (spike N501Y). Combining UDG-RT-LAMP technology, CRISPR-LAMP design, and mutation detection design, we developed a CRISPR-LAMP detection platform that can precisely diagnose pathogens with better stability and significantly improved point mutation detection efficiency.



INTRODUCTION

With the exploding of COVID-19, rapid, inexpensive, and accurate diagnosis of pathogens is an urgent need to prevent another public health emergency.¹ Nucleic acid point of care testing (POCT) has been widely used in pathogen diagnosis, especially for those who require fast and accurate tests, like respiratory tract infections.^{2,3} Loop-mediated amplification (LAMP) technology is an isothermal amplification technology that stands out in both research and industrial areas for its cost, sensitivity, robustness, accessibility, and so on.⁴ Although LAMP has a high amplification efficiency, false-positive results always influence diagnosis interpretation.⁵ A universally stable isothermal LAMP system (with low false-positive) is necessary for precise diagnosis. Clustered, regularly interspaced, short palindromic repeats (CRISPR) based diagnostics (CRISPRDx) has been combined into practice among many isothermal systems.^{6–8} These programmable endonucleases and mature CRISPR RNA (crRNA) complexes can produce trans-cleavage signal origin from template recognition-dependent cis-cleavage which can be combined with the isothermal amplification technology.^{9–11} Uracil-DNA-glycosylase (UDG) can efficiently clear uracil bases both in double-stranded and

single-stranded DNA which does not affect natural DNA.¹² Replacing deoxythymidine triphosphate (dTTP) with deoxyuridine triphosphate (dUTP) in the polymerase chain reaction (PCR) and LAMP, amplicons incorporated with uracil bases can be efficiently digested which can be utilized in preamplification treatment to eliminate carryover of the previous dUTP-incorporated aerosol.^{13,14} Although CRISPR-LAMP technology has been combined successfully in POCT for nucleic acid detection,^{15,16} there are a few detailed studies about its' constraints and application field, especially where single LAMP technology cannot work well.

Respiratory tract viruses are a widely spread pathogen that always explodes between winter and spring and can result in life-threatening lower respiratory tract illness.¹⁷ Among these,

Received: September 18, 2021

Accepted: November 12, 2021

Published: November 25, 2021



influenza A virus (FLUA), influenza B virus (FLUB), respiratory syncytial A virus (RSVA), and respiratory syncytial B virus (RSVB) are among the major accompanied respiratory tract viruses. According to Shanghai East Hospital's unpublished statistical data, 1023 cases (of 8456 collected cases from nasopharyngeal swabs specimens) were influenza A virus infected, 379 cases (of 8456 collected cases from nasopharyngeal swabs specimens) were influenza B virus infected, and 14 cases (of 1720 collected cases from blood specimens) were respiratory syncytial virus infected from December 1, 2018 to July 31, 2019. Influenza is a zoonic, highly contagious, and acute respiratory disease whose clinical symptoms range from mild cough, high fever, and headache to life-threatening pneumonia.¹⁸ The main threat of respiratory syncytial virus infection was bronchiolitis and pneumonia.¹⁹ With the development of COVID-19, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may be incorporated into the common respiratory tract virus. So, precise POCT diagnosis of these five pathogens is an urgent need. SARS-CoV-2 B.1.1.7 variant was reported first by the United Kingdom which increased SARS-CoV-2 transmission and has been detected in over 30 countries.²⁰ For the B.1.1.7 variant, spike N501Y (A23063T in nucleic acid) is the main mutation point that needs rigorous monitoring.²¹ Like amplification refractory mutation system PCR technology, LAMP technology can distinguish between wild-type and mutant type by utilizing highly specific primers with the mutation in the location of amplification-inhibited sites.^{22,23} However, the discrimination was always a delay of time to positive (TTP) which was not obvious at a lower concentration of the template approaching the limit of detection (LoD).²⁴ A precise and sensitive strategy for LAMP technology to detect point mutation urgently needs to be supplemented.

To solve the shortage of these five pathogen POCT diagnoses, including mutant SARS-CoV-2 spike N501Y, and at the same time develop a universally stable isothermal amplification platform (with low false-positive and high signal-background ratio), we developed a universally stable and precise CRISPR-LAMP detection platform (UCLD). There were several problems to be solved: (1) What is the influence of an extra introduced dUTP nucleotide on fluorescence LAMP and CRISPR-LAMP technology? (2) Are there position constraints on crRNA design with complex structured LAMP amplicons? (3) Comparison of sensitivity between fluorescence LAMP and CRISPR-LAMP technology. (4) Mutation detection efficiency with LAMP technology and CRISPR-LAMP technology. We found that an extra introduced dUTP nucleotide not only improves UDG cleavage efficiency but also increases the stability of this isothermal amplification system (both in fluorescence LAMP and CRISPR-LAMP technology, with low false-positive). Compared with fluorescence LAMP, the CRISPR-LAMP technology can even improve sensitivity to 10 times. The crRNA efficacy was not influenced at positions all around F2–B2 of LAMP amplicons, even the stem-loop, and thus greatly enlarges crRNA design efficacy. Unlike the LAMP mutation detection strategy, CRISPR-LAMP technology can efficiently detect point mutation with two means (wild-type detect and mutant under detect; wild-type under detect and mutant detect) at 10 copies/ μL at a 1% ratio of background (wild-type + mutant). This UCLD platform can advance the CRISPR-LAMP technology with more accuracy, more stability, and more sensitivity, providing more complete

and easier CRISPR-LAMP design methods and simultaneously making it more easy and precise to detect point mutation.

■ EXPERIMENTAL SECTION

Primers and crRNA Design. According to our sequence analysis, we chose a relatively conserved sequence for LAMP primer design based on web tools (<http://primerexplorer.jp/e/index.html>). FLUA H1 subtype HA gene, FLUA-H3 subtype HA gene, FLUB NS1 and NEP genes, RSVA M gene, RSVB G gene, and SARS-CoV-2 N and RdRp gene were chosen to design LAMP primers. After several runs of screening of sensitivity and specificity, we chose the best primer pairs to perform the LAMP assay. Detailed LAMP primer sequence of these six pathogens of seven gene targets is shown in Table S4. For the spike N501Y general LAMP primer design, point mutation sites should be put between F2–F1, F1–B1c, or B2c–B1c sites. General LAMP primers are shown in Table S4. Confirming the best LAMP primer pairs, we select the crRNA spacer sequence or the complementary sequence in the LAMP F2–B2 region. TTTV PAM sites are necessary, and a 20 nt spacer sequence was preferred.²⁵ After all spacer sequences were found, mFold²⁶ web tools (<http://www.unafold.org/mfold/applications/rna-folding-form.php>) were used to calculate the RNA structure. Correct stem-loop structure in the direct repeat was necessary, and the first 5 nt on the target specific sequence should be free. Besides, primer sequence disturbs and spacer sequence cross-interference with other pathogens should be avoided with NCBI BLAST web tools (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Structure optimized sequence can then be optimized by the Cas12a in vitro cleavage reaction. For point mutation detection, the point mutation site should be designed between 1st and –16 th base positions of the spacer sequence²⁷ and calculated with wild-type and mutant. All spacer sequences are shown in Table S4.

crRNA Synthesis and Screen. Forward *Lachnospiraceae bacterium ND2006* (Lba) Cas12a sequence (/5/AATTCTAA-TACGACTCACTATAGGTAATTTCTACTAAGTGTA-GAT/3/) and reverse LbaCas12a sequence (/5/spacer sequence complementary + ATCTACACTTAGTAGAAAT-TACC/3/) were annealed and PCR amplified. Then, purified PCR amplicons were transcribed with the HiScribe T7 high-yield RNA synthesis kit for 16 h at 37 °C. Afterward, the monarch RNA cleanup kit was used to purify crRNA. Transcribed crRNAs were quantified with the Qubit FLEX RNA HS assay and stored at –80 °C for later use. The target gene sequences of FLUA-H1, FLUA-H3, FLUB, RSVA, RSVB, SARS-CoV-2 N and RdRp, and rhinovirus were inserted into the pUC57 plasmid and then PCR-amplified with M13 primers. Then, purified PCR amplicons were transcribed with the HiScribe T7 high yield RNA synthesis kit for 2 h at 37 °C, and afterward, the monarch RNA cleanup kit was used to purify the transcribed RNA. RNA templates were quantified with the Qubit FLEX RNA BR assay. PCR template was amplified with F3/B3 primer pairs of each LAMP primer pair with synthesized plasmids, purified with the QIAquick Gel Extraction Kit, and then quantified by the Qubit FLEX dsDNA BR assay. A final concentration of 20 nM DNA was put into the Cas12a cleavage reaction to calculate the cleavage efficiency. 10⁶ copies of templates were put into dTTP LAMP and dUTP LAMP reactions, respectively, and then 2 μL of amplicons were put into the Cas12a cleavage reaction.

UDG-RT-LAMP Assay and Cas12a Cleavage Assay. 10⁶ copies of the rhinovirus template were utilized to calculate the

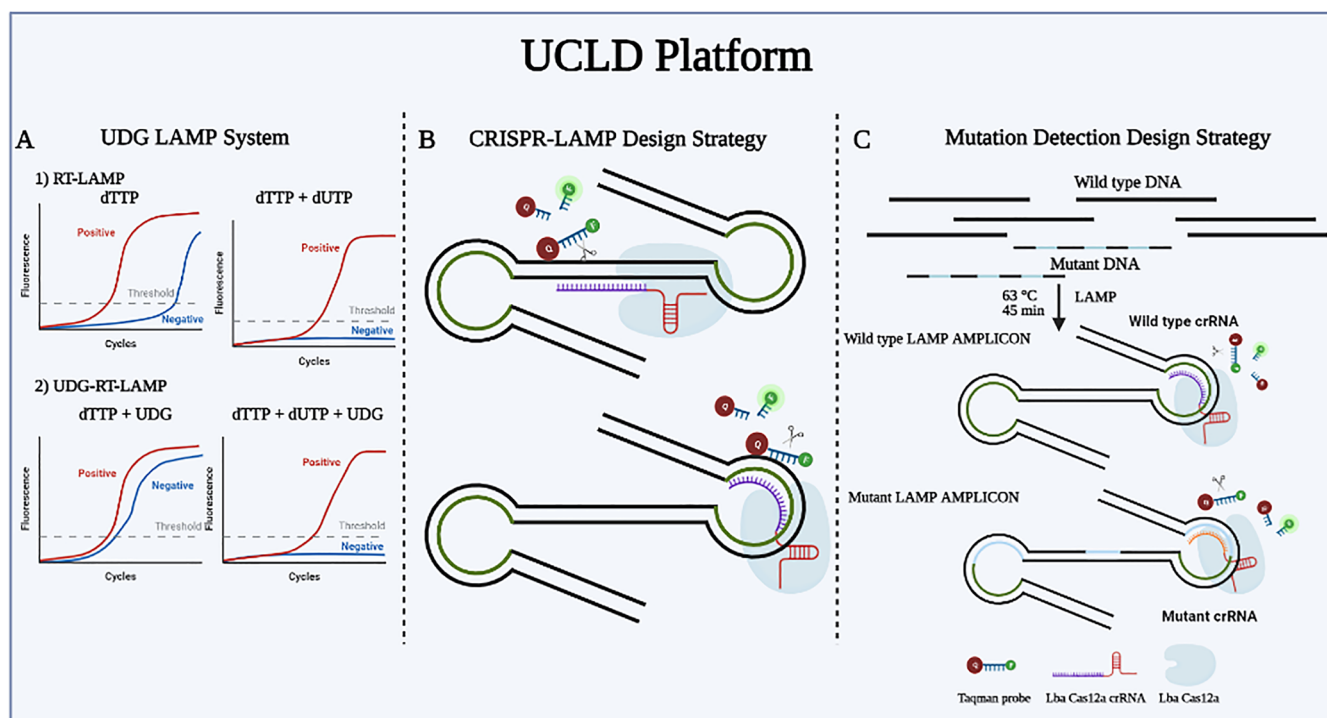


Figure 1. Overview of the UCLD Platform. (A) UDG-RT-LAMP system was developed with the addition of dUTP. (1) dUTP itself can reduce false-positive rate in the LAMP system. (2) A dUTP incorporated amplicon can be efficiently removed with the UDG enzyme. (B) CRISPR guide RNA can be designed anywhere among LAMP amplicon F2–B2 areas without position constraints. (C) Point mutation detection strategy was enlarged with general LAMP amplification combined with Cas12a mismatch recognition in two means (wild-type detect, mutant under detect and mutant detect, wild-type under detect). This picture was created with [BioRender.com](https://www.biorender.com).

clearing aerosol contamination ability. First, five ratios of dUTP/dTTP were put into the RT-LAMP reaction (without SYBR Green I) for 60 min at 63 °C. Then, the first round of LAMP amplicons was diluted 1000 times and 100,000 times, and the UDG enzyme was used to calculate the clearing ability of the simulated aerosol. Besides rhinovirus, a bocavirus template was also utilized to test this influence. Rhinovirus LAMP primers are shown in [Table S4](#). For the standard Cas12a cleavage assay, 50 nM LbaCas12a was preincubated with 50 nM crRNA in 1× NEB buffer 2.1 for 10 min at 25 °C. Then, taqman reporter (5'-FAM/TTATTATT/3'-BHQ-1/) was added to the reaction at a final concentration of 500 nM. Afterward, 2 μL of template (PCR amplicons or LAMP amplicons) was added into the reaction for 45 min at 37 °C.

Other materials and detailed methods are shown in the supplementary file.

RESULTS AND DISCUSSION

UCLD Platform. False-positive or an early TTP of the negative test was a big problem to verify the LAMP system.⁵ Aerosol contamination and primer stability may be the leading cause. We first developed a UDG-RT-LAMP system that can efficiently remove LAMP amplicons ([Figure 1A-2](#)). Besides, we found that an extra dUTP itself in the LAMP system can reduce the false-positive rate ([Figure 1A-1](#)). We guessed that an extra dUTP can slow down the primer polymerization rate and thus reduce false-positive rates (fluorescence rising rate of positive reaction decreased similarly, but there was a less delay of the TTP of positive reaction). We then utilized combined CRISPR-LAMP technology to advance LAMP technology, and both sensitivity and stability were improved. For Cas12a crRNA design, nowadays, there are less mature design tools.

Design tools, like Cas-OFFinder,²⁸ CRISPOR,²⁹ CHOP-CHOP,³⁰ and et al., are easier operated for in vivo cleavage assay, not suitable for in vitro assay, which need additionally considering primer cross-reaction interference. For LAMP amplicons, there were doubts about the crRNA design constraints for the special stem-loop structured amplicons. Twenty-eight crRNAs were designed to rule out interference ([Figure 1B](#)). In addition, LbaCas12a/crRNA cleavage background still needs to be overcome to improve sensitivity. From our results, although some crRNAs have some fluorescence background in the Cas12a cleavage assay, they did not influence result interpretations, for the fluorescence of positive results was significantly higher than that of negative results. Reports have shown that variation between NEB buffer 3.1 and NEB buffer 2.1 can change the background in RPA-Cas12a.³¹ From our study, this was not a major factor in our proposed systems. We found that the real concentration of crRNA may influence the background ([Figure S1B](#)). Owing to crRNA always having different structures, the real concentration of effector crRNA was especially important. We compared the concentration of crRNA measured by a Bio-Rad Spectrophotometer and a Qubit FLEX fluorometer and found there was about 3 times difference. For different batches of crRNAs, a series of concentrations can be tested to minimize the background. This UCLD system also proposed two means to discriminate point mutation (wild-type detect, mutant under detect and mutant detect, wild-type under detect) which greatly enlarge point mutation detection efficiency ([Figure 1C](#)). Although many portable and visual Cas12a platforms have been developed,^{32–34} two-step reactions (target amplification followed by a CRISPR based detection) are still more accurate and have been coupled with dipsticks for POCT.³⁵

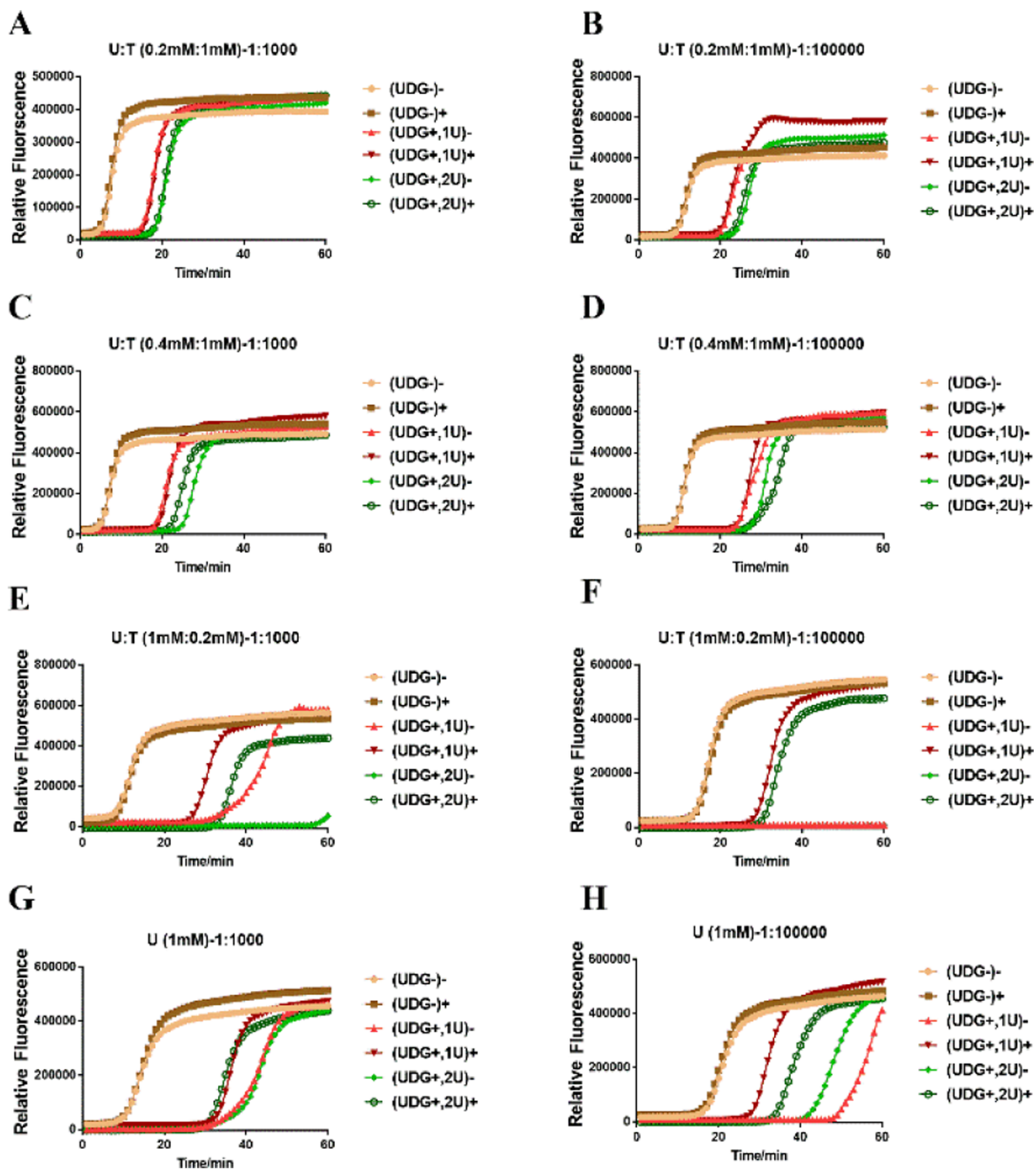


Figure 2. LAMP amplicons removing the ability of different dUTP/dTTP concentration ratios. For different groups: “-” inside brackets means the UDG enzyme was absent and “+” inside brackets means the UDG enzyme was present. “-” outside brackets means template was absent and “+” outside brackets means template was present.

Our proposed UCLD platform combined UDG-RT-LAMP system (avoid aerosol contamination), CRISPR-LAMP design strategy, and point mutation detection strategy together which can advance CRISPR-LAMP technology to more accurate, stable, and sensitive, expanding its application, especially in point mutation detection, which makes it to be used more easily in precise POCT.

Establishment of UDG-RT-LAMP System. To prevent aerosol contamination from LAMP-CRISPR combined technology, we first established a UDG-RT-LAMP system. Once dUTP is incorporated into LAMP amplicon, UDG can efficiently remove uracil-containing DNA without affecting natural DNA. So, the carryover of previous amplification can be efficiently removed in the UDG-RT-LAMP system before

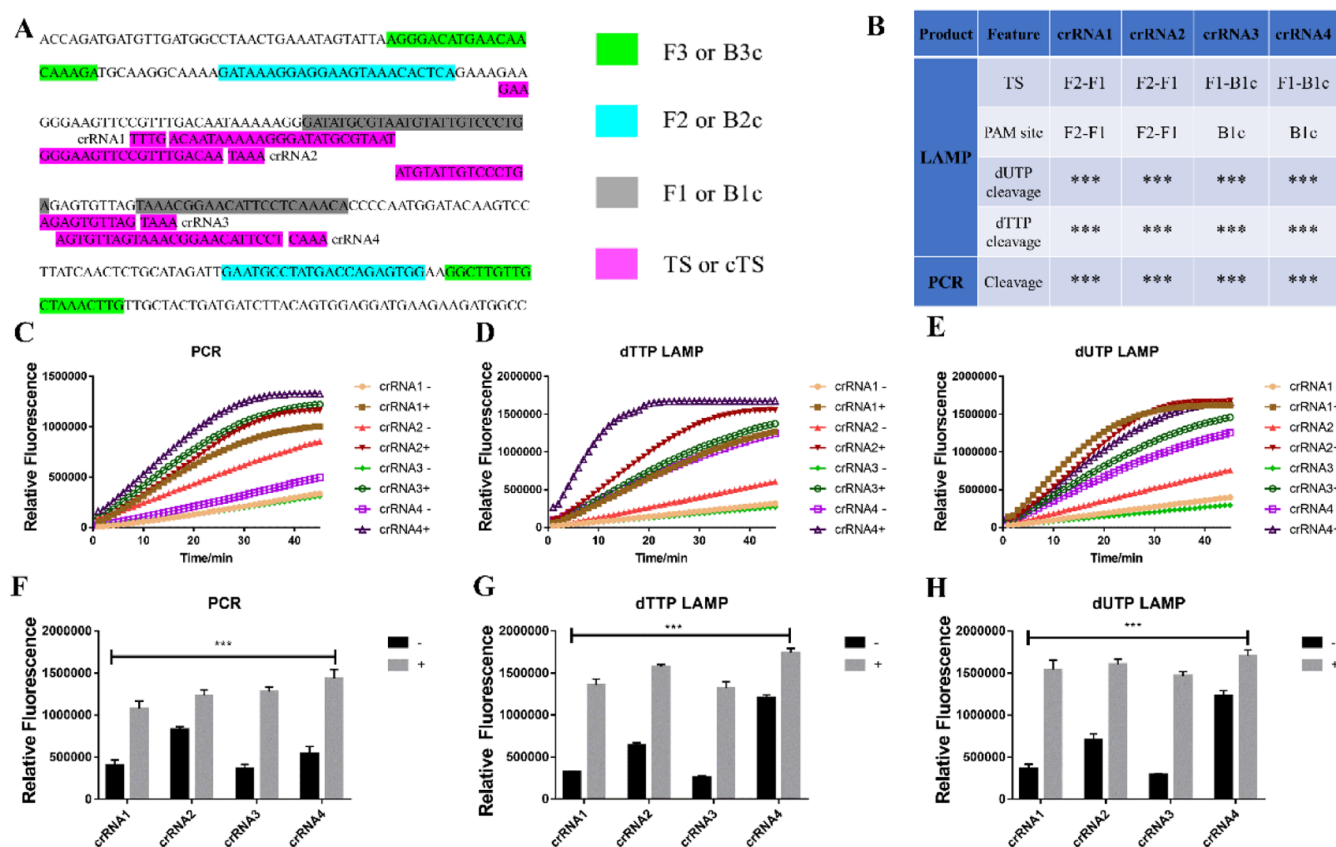


Figure 3. FLUB crRNA screen and cas12 cleavage assay. “***” means there was a significant difference between the blank (without product) and test (with product) group ($P < 0.001$). “-” means this type of amplicon was absent. “+” means this type of amplicon was present. (A) FLUB LAMP primer and crRNA anchoring sites. (B) Statistical data of crRNA anchoring sites and cleavage efficiency with linearized PCR amplicons, dTTP LAMP amplicons, and dUTP LAMP amplicons. TS = target strand. (C) Cleavage efficiency of linearized PCR amplicons with LbaCas12a/structure-optimized crRNA. (D) Cleavage efficiency of dTTP LAMP amplicons with LbaCas12a/structure-optimized crRNA. (E) Cleavage efficiency of dUTP LAMP amplicons with LbaCas12a/structure-optimized crRNA. (F) Endpoint fluorescence of cleavage assay of linearized PCR amplicons with LbaCas12a/structure-optimized crRNA ($n = 3$ technical replicates; bars represent mean \pm SD). (G) Endpoint fluorescence of cleavage assay of dTTP LAMP amplicons with LbaCas12a/structure-optimized crRNA ($n = 3$ technical replicates; bars represent mean \pm SD). (H) Endpoint fluorescence of cleavage assay of dUTP LAMP amplicons with LbaCas12a/structure-optimized crRNA ($n = 3$ technical replicates; bars represent mean \pm SD).

current amplification. However, dUTP-incorporated dNTP mixture influenced the amplification rate, and a balance should be obtained between the aerosol contamination clearing ability and the amplification rate. Preamplified amplicons were utilized to stimulate carryover contamination, and five ratios of dUTP/dTTP concentrations were compared with their amplicons contamination clearing ability (Figure 2) and amplification rate (Figure S2), and a final concentration of 1 mM dUTP: 0.2 mM dTTP, not a 1 mM dUTP, was shown to have a stable aerosol clearing ability with an acceptable 10–15 min delay of TTP. With the rising concentration of dUTP in the dNTP mixture, UDG (2U) can efficiently remove preamplified amplicons without affecting current amplification (negative: no C_t value; positive: less delayed C_t value). Results (Figure 2) also found that replacing dTTP totally with dUTP did not improve UDG clearing ability (this was observed in both rhinovirus and bocavirus UDG-RT-LAMP reaction), and we guessed that a small amount of dTTP may increase the amount of dUTP-incorporated LAMP amplicons which were easily removed by UDG. Focusing on common human susceptible respiratory tract virus types/subtypes, we established a typical LAMP system on six types/subtypes of virus with seven gene targets with high sensitivity and specificity

(Figures S3 & S4). Compared with a typical RT-LAMP system (dTTP LAMP), the UDG-RT-LAMP system (dUTP LAMP) almost does not influence sensitivity (except for RSVB target) and specificity (Figures S5 & S6). Referring to the previous UDG-PCR system and UDG-RT-LAMP system, the proposed UDG-RT-LAMP system has the same antiaerosol contamination ability.^{14,36} Additional discovery was that an extra incorporated dUTP nucleotide can reduce the false-negative rate (data not shown) originated from unstable primer pairs (primers with good sensitivity but can produce uncertain false-positive results after using for a period).

Universal CRISPR-LAMP Design. After confirming the dUTP LAMP system, we combined this system with CRISPR-LAMP technology to optimize the crRNA design. Concentrations of Cas12a: crRNA were optimized to 50 nM: 50 nM which has the rising trend of background-subtracted fluorescence with time (Figure S1). Cas12a cleavage assay showed that almost all structure-optimized crRNA (analyzed by secondary structure: Correct stem-loop structure in the direct repeat between positions 6 and 20 was necessary, and the first 5 nt on the target specific sequence should be free) have cleavage activity (Figure 3). However, PCR amplicons of the LAMP F3/B3 area can trigger more crRNAs' cleavage

activity than dTTP LAMP and dUTP LAMP amplicons (Figures S7–S12). Statistical data (Figure 4B) showed that

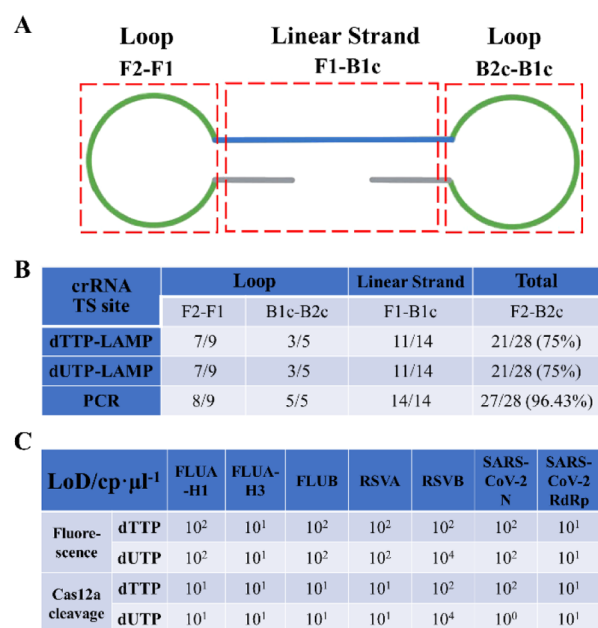


Figure 4. crRNA design strategy and cleavage efficiency. (A) Illustration of the initial LAMP stem-loop product. Red rectangle labeled areas were three candidate target positions for crRNA binding. (B) Statistical data of crRNA cleavage efficiency from Figures 2 and S8–S13. (C) LoD between fluorescence LAMP and CRISPR-LAMP on six types/subtypes of virus with seven gene targets.

27/28 LbaCas12/crRNAs have cleavage activity with linearized PCR amplicons, and 21/28 LbaCas12/crRNAs have cleavage activity with dTTP LAMP and dUTP LAMP amplicons. FLUA-H3 crRNA1 has no cleavage activity among these three amplification amplicons, which means that this crRNA did not form the correct structure. Except for this crRNA, there were another six crRNAs which have no cleavage activity in both dTTP LAMP and dUTP LAMP amplicons, and there were no target position restrictions, for one crRNA mainly binds the F2–F1 position (looped-strand), two crRNAs mainly bind the B1c–B2c position (looped-strand), and three crRNAs mainly bind the F1–B1c position (linearized-strand). This means when using CRISPR-LAMP technology, using linearized PCR amplicons to screen crRNA may cause false-positive results. The initial LAMP amplicons were a double stem-loop structure (Figure 4A) and then form numerous loop–strand–loop structured double-stranded amplicons, where F2–F1 and B2c–B1c areas were looped double strands and F1–B1c areas were linearized strands. Excluding FLUA-H3 crRNA1, the results (Figure 4B) showed that crRNA can bind looped double strands (7/8 crRNAs on the F2–F1 loop and 3/5 crRNAs on the B1c–B2c loop) which were similar to linearized strands (11/14 crRNAs on F1–B1c linearized strands). These results have shown that crRNA designed anywhere between F2 and B2c positions can achieve high cleavage efficacy. Because the first 5 nt on the target specific sequence is the seed sequence of Cas12a crRNA and is essential for crRNA to bind the target,^{37,38} it is postulated that the crRNA binding sites determine its Cas12a cleavage efficiency. With these seven gene targets, we designed structure-optimized crRNAs as many as possible to cover all

locations between F2 and B2c as fully as possible and found that these rules and afterward confirm their generalizability, which has not been described before. For CRISPR-LAMP technology, there were combination possibilities: (1) Best LAMP primers (high LoD and specificity) + best crRNA (high signal-background ratio); (2) best LAMP primers (high LoD and specificity) + poor crRNA (low signal-background ratio); (3) best crRNA (high signal-background ratio) + poor LAMP primers (low LoD and specificity). With our proposed crRNA design strategy, almost double number of crRNAs can be optimized with LAMP amplicons, which greatly improved the crRNA design accuracy. In addition, we also found that there were occasionally existing some different cleavage results between dTTP LAMP amplicons and dUTP LAMP amplicons and that some crRNAs have no cleavage activity on dTTP LAMP amplicons while having a high cleavage activity on dUTP LAMP amplicons (10 times of 28 independent tests) (data in preparation). These unstable results mean that dUTP LAMP amplicons have stable cleavage activity, and combined CRISPR-LAMP technology could significantly decrease the false-negative rate of crRNA screening.

Comparison of Sensitivity Between LAMP and CRISPR-LAMP Technology. We compared the sensitivity of the dTTP LAMP combined CRISPR-LAMP assay with the dUTP LAMP combined CRISPR-LAMP assay. Results (Figure S13) show that there was almost no difference between these two assays, except for the RSVB target which may be because the dUTP LAMP system influenced the amplification rate with this target within 45 min. Optimized crRNAs after screening for the same target showed no discrimination in LoD, and the only difference was the Δ fluorescence intensity. Specificity assay results (Figure S14) show that both dTTP LAMP combined CRISPR-LAMP assay and dUTP LAMP combined CRISPR-LAMP assay have no cross-interference. Compared with fluorescence LAMP, CRISPR-LAMP technology can even increase sensitivity to 10 (Figure 4C). LAMP reaction near the limit was unstable which may produce insufficient amplification signal collected by a fluorescence reader. We have tested that about 1 nM final concentration of template can achieve cleavage activity in the in vitro Cas12a cleavage assay (data not shown) without preamplification, and this means that about 10⁹ copies of amplicons can achieve a high Cas12a cleavage efficiency. These results have shown that maybe some under detect amplicons with fluorescent LAMP technology may be detected with CRISPR-LAMP technology. Besides, some amplifications have not reached exponential progress in a typical 45 min LAMP reaction, while these accumulated amplicons may be cleaved by the CRISPR/Cas12a complex. To further test this CRISPR-LAMP technology, we utilized 12 positive and 6 negative samples to calculate the proposed UCLD platform (Figures S15–S18). Original results are shown in Table S1, and statistical data are shown in Table S2. Three different polymerases were utilized to compare the stability and sensitivity. Results show that the Bst 2.0 Warmstart amplification system has the best stability and sensitivity, and the Bst 3.0 amplification system has the worst. Having better stability and amplification rate,³⁹ the Bst 2.0 Warmstart polymerase amplification system performed well than the Bst LF amplification system and so as its CRISPR-LAMP system. Although Bst 3.0 DNA polymerase has strong strand displacement activity and high reverse transcriptase activity,⁴⁰ its amplification system performed the worst for higher false-positive amplicons which reduced sensitivity in

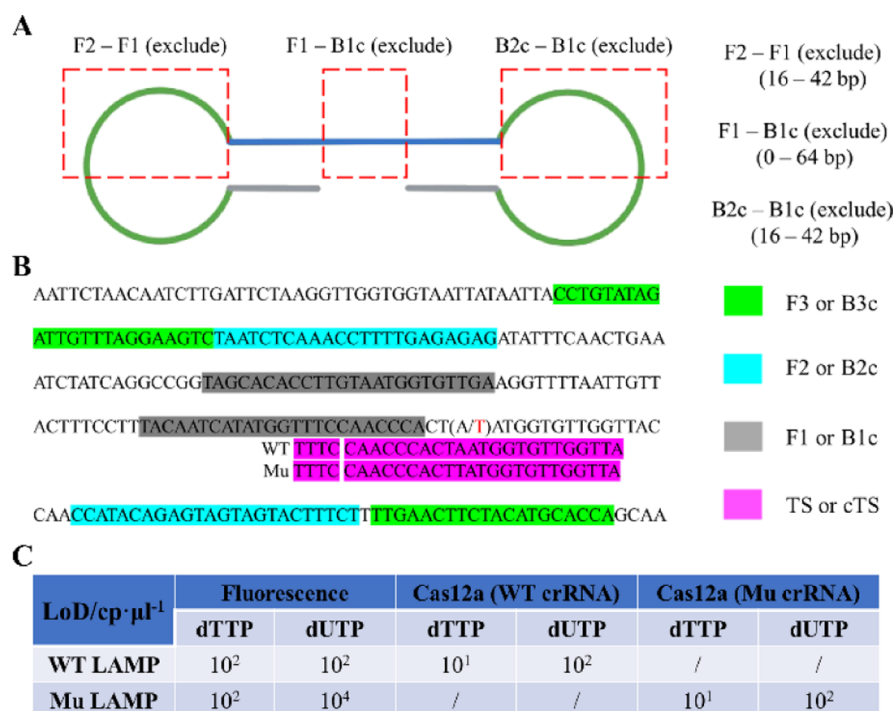


Figure 5. Point mutation detection with UCLD technology. (A) Illustration of LAMP primer design strategy for point mutation detection. Red rectangle labeled areas were three candidate target positions for point mutation sites. (B) LAMP primer and crRNA anchoring sites of SARS-CoV-2 B.1.1.7 variant major genotype. The red word inside the bracket was the major point mutation nucleotide, while another was the wild-type. (C) Statistical data of the wild-type and spike N501Y detection efficiency with prolonged LAMP reaction.

both LAMP and CRISPR-LAMP systems. The best dTTP LAMP combined CRISPR-LAMP assay showed 83.33% (10/12) sensitivity and 100% (6/6) specificity, and the best dUTP LAMP combined CRISPR-LAMP assay showed 75% (9/12) sensitivity and 100% (6/6) specificity, and the RSVB-infected samples was the leading reason for the sensitivity decrease. Except for the Bst 3.0 amplification system, all CRISPR-LAMP systems showed higher sensitivity than their corresponding LAMP amplification system. For the Bst 3.0 amplification system, the CRISPR-LAMP system showed higher specificity than its corresponding LAMP amplification system. The clinical CRISPR-LAMP results were consistent with that of our prepared templates that the CRISPR-LAMP technology can significantly improve sensitivity and stability compared with single LAMP technology. Although the ULC platform has been reported to utilize dUTP to replace dTTP to achieve single-copy sensitivity in Cas12a cleavage,⁴¹ the difference between dUTP systems and dTTP systems (except for aerosol contamination) in CRISPR-LAMP technology still needs a more detailed study.

Mutant SARS-CoV-2 Spike N501Y Detection Design.

To enlarge the application of this CRISPR-LAMP technology, we expanded the application of CRISPR-LAMP technology in point mutation detection, which is shown in Figure 5A. Using common LAMP primers, both wild-type and mutant can be amplified and afterward detected with corresponding wild-type crRNA and mutant crRNA. LAMP technology always utilized highly specific primers with the mutation in the locations at the primer FIP F1c 5' term (BIP B1c 5' term), FIP F2 3' term (BIP B2 3' term), and F3 3' term (B3 3' term)⁴² to discriminate wild-type and mutant type, of which these six primer sites were essential to form initial LAMP stem-loop amplicons (http://loopamp.eiken.co.jp/e/lamp/snps_index.html).

However, the Δ TTP between the wild-type and mutant type was so small and not sensitive enough. Here, we focus on spike N501Y mutation and compared the LAMP technology with the CRISPR-LAMP technology. However, specific LAMP primers either for wild-type or spike N501Y cannot be designed. With CRISPR-LAMP technology, we designed common LAMP primers, wild-type crRNA, and spike N501Y crRNA which are shown in Figure 5B. Both dTTP and dUTP systems have the same trend for wild-type and spike N501Y detection (Figure S20). Two means were presented: (1) wild-type detect and mutant under detect with wild-type crRNA which was useful for those unclear mutation sites (1 → 3); (2) wild-type under detect and mutant detect with mutant crRNA which was useful for those clear mutation sites (1 → 1). Comparison of our proposed general LAMP amplification efficiency and point mutation detection efficiency is shown in Figures S20 & S21. Statistical data are shown in Table S3 that proposed CRISPR-LAMP technology can improve sensitivity to 100 times than LAMP technology and can precisely detect wild-type and spike N501Y, respectively. However, the point mutation detection sensitivity was not good, especially for the dUTP amplification system. So, we doubled the LAMP reaction time to calculate the influence on the sensitivity of LAMP and point mutation detection efficiency. LAMP sensitivity corrected by melt curve significantly improved sensitivity, especially for the dUTP LAMP system (Figure S22). CRISPR-LAMP technology can detect trace amounts of amplified template, which was invisible in the LAMP technology, even in the corrected melt curve plot (Figure S23). Doubling the LAMP reaction time, CRISPR-LAMP technology can even achieve 10 cp/μL (10 copies of template per reaction) in wild-type and spike N501Y detection (Figure 5C). This means with full amplification reaction, the dUTP system

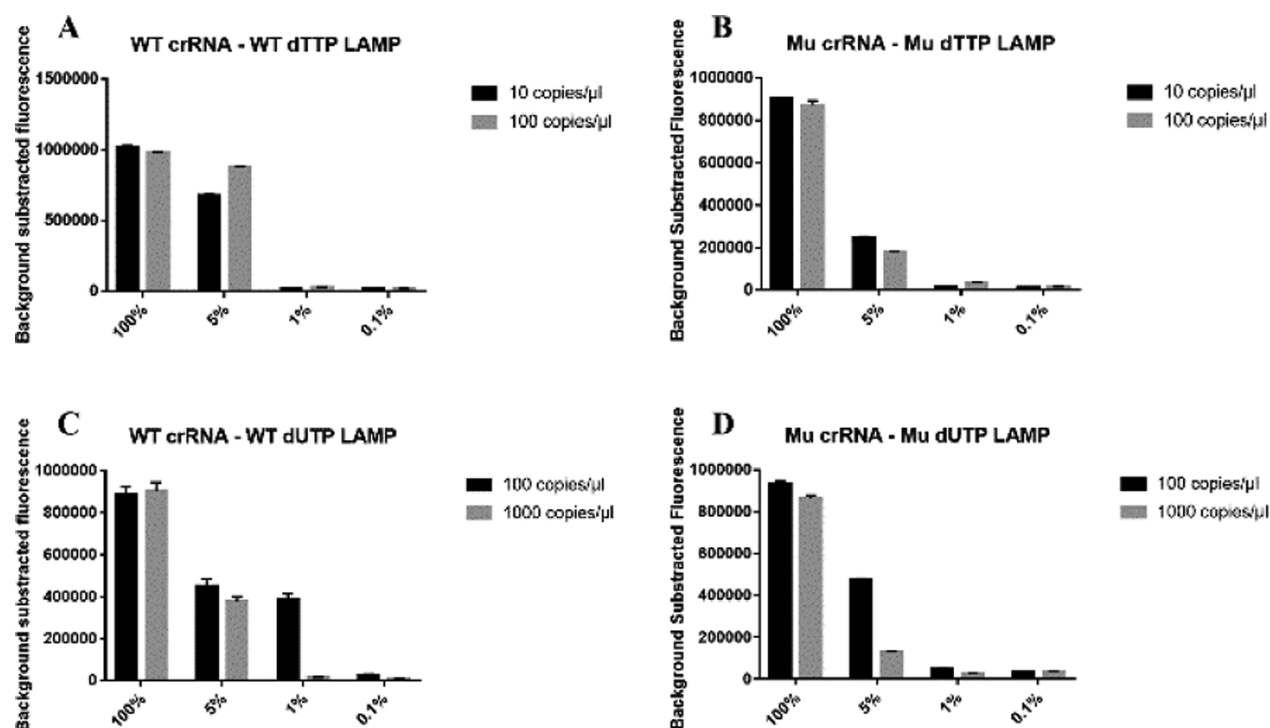


Figure 6. Detection of wild-type or spike N501Y as a fraction of the background target. (A) dTTP LAMP reaction was combined with CRISPR-LAMP assay using wild-type crRNA. (B) dTTP LAMP reaction was combined with CRISPR-LAMP assay using spike N501Y crRNA. (C) dUTP LAMP reaction was combined with CRISPR-LAMP assay using wild-type crRNA. (D) dUTP LAMP reaction was combined with CRISPR-LAMP assay using spike N501Y crRNA.

can achieve the same sensitivity as the dTTP system, which may be useful to improve the sensitivity of the dUTP system, like our proposed RSVB dUTP system. We also found that CRISPR-LAMP technology can detect wild-type on the background of spike N501Y at the level of 1% (Figure 6). Although single nucleotide polymorphism (SNP) detection with CRISPR technology has been reported, like HOLMESv2 platform⁴³ with Cas12b combined technology (not clear with combined LAMP technology) and SHERLOCK platform⁷ with RPA-Cas13 combined technology, utilizing CRISPR-LAMP technology to detect point mutation has not been clearly reported before, which can detect both wild-type and mutant. This CRISPR-LAMP technology can efficiently detect point mutation which single LAMP technology cannot solve and has great potential in (SNP) detection for its good allele disturbing ability.

CONCLUSIONS

Herein, we proposed a UCLD platform that can advance CRISPR-LAMP technology with improved sensitivity and stability. We developed a CRISPR-LAMP technology to detect FLUA, FLUB, RSVA, RSVB, and SARS-CoV-2. UDG-RT-LAMP was also developed and incorporated into CRISPR-LAMP technology. The LoD can be even 1 copies/ μ L of template, and stability was significantly improved. A CRISPR-LAMP assay for SARS-CoV-2 B.1.1.7 variant major point mutation spike N501Y has also been developed. For LAMP amplicons, crRNA can be designed anywhere without position constraints. Combined with common LAMP primers, CRISPR-LAMP technology can efficiently detect point mutation with 10 copies of template which can even detect wild-type at 1% levels on the background of spike N501Y template. For CRISPR-LAMP technology, a precisely liquid

controlling chip may be a useful platform for two-step operations, which needs further study. We are now testing a fully automated chip which can integrate CRISPR-LAMP technology into a single chip without additional labor and thus decrease the risk of aerosol contamination. Besides, a new widely spread variant of concern of SARS-CoV-2 (B.1.617.2) has recently appeared through the globe, and we have utilized our UCLD platform to monitor the important point mutation. A more automated, sensitive, and stable UCLD platform can be improved to enlarge CRISPR-LAMP application.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.1c04065>.

Materials, point mutation detection strategy, detailed validation on LAMP, CRISPR-LAMP, and UDG-RT-LAMP systems, clinical original results, original primers, and crRNA sequence (PDF)

AUTHOR INFORMATION

Corresponding Authors

Lieying Fan – Department of Clinical Laboratory, Shanghai East Hospital, School of Medicine, Tong Ji University, Shanghai 200120, P. R. China; Email: flieying@yeah.net

Guodong Sui – Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP3), Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, P. R. China; Department of Medical Microbiology and Parasitology, School of Basic Medical Sciences, Fudan University, Shanghai 200032, P. R. China; Jiangsu Collaborative Innovation Center of Atmospheric

Environment and Equipment Technology (CICAEET), Nanjing University of Information Science & Technology, Nanjing 210044, PR China; orcid.org/0000-0002-6751-0852; Email: gsui@fudan.edu.cn

Authors

Tong Zhang – Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP3), Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, P. R. China

Wei Zhao – Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP3), Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, P. R. China

Wang Zhao – Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP3), Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, P. R. China

Yuying Si – Department of Clinical Laboratory, Shanghai East Hospital, School of Medicine, Tong Ji University, Shanghai 200120, P. R. China

Nianzhen Chen – Department of Clinical Laboratory, Shanghai East Hospital, School of Medicine, Tong Ji University, Shanghai 200120, P. R. China

Xi Chen – Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP3), Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, P. R. China

Xinlian Zhang – Shanghai Key Laboratory of Atmospheric Particle Pollution and Prevention (LAP3), Department of Environmental Science and Engineering, Fudan University, Shanghai 200433, P. R. China

Complete contact information is available at:

<https://pubs.acs.org/10.1021/acs.analchem.1c04065>

Author Contributions

T.Z., W.Z., and W.Z. contributed equally to this work. T.Z.: conceptualization, methodology, data curation, validation, and writing the original draft. W.Z.: data curation, validation, and conceptualization. W.Z.: resources, investigation, and conceptualization. Y.S.: data curation and validation. N.C.: resources and investigation. X.C.: instrument operation. X.Z.: supervision. L.F.: conceptualization and funding acquisition. G.S.: conceptualization, funding acquisition, and writing—review and editing.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This work was supported by the Shanghai Science and Technology Commission (19441903700, 21DZ1202300), the COVID-19 research project for universities (Ministry of Education), the novel coronavirus research project of Fudan University, and Major Project of Basic Research of Shanghai Science and Technology Commission (17JC1401002).

REFERENCES

- (1) Nelson, P. P.; Rath, B. A.; Fragkou, P. C.; Antalis, E.; Tsiodras, S.; Skevaki, C. *Front. Cell. Infect. Microbiol.* **2020**, *10*, 181.
- (2) Egilmez, E.; Walker, G. J.; Bakthavathsalam, P.; Peterson, J. R.; Gooding, J. J.; Rawlinson, W.; Stelzer-Braid, S. *Rev. Med. Virol.* **2018**, *28*, No. e1995.

(3) Tsao, Y.-T.; Tsai, Y.-H.; Liao, W.-T.; Shen, C.-J.; Shen, C.-F.; Cheng, C.-M. *Trends Mol. Med.* **2020**, *26*, 1118–1132.

(4) Moehling, T. J.; Choi, G.; Dugan, L. C.; Salit, M.; Meagher, R. J. *Expert Rev. Mol. Diagn.* **2021**, *21*, 43–61.

(5) Schneider, L.; Blakely, H.; Tripathi, A. *Electrophoresis* **2019**, *40*, 2706–2717.

(6) Broughton, J. P.; Deng, X.; Yu, G.; Fasching, C. L.; Servellita, V.; Singh, J.; Miao, X.; Streithorst, J. A.; Granados, A.; Sotomayor-Gonzalez, A.; Zorn, K.; Gopez, A.; Hsu, E.; Gu, W.; Miller, S.; Pan, C.-Y.; Guevara, H.; Wadford, D. A.; Chen, J. S.; Chiu, C. Y. *Nat. Biotechnol.* **2020**, *38*, 870–874.

(7) Gootenberg, J. S.; Abudayyeh, O. O.; Lee, J. W.; Essletzbichler, P.; Dy, A. J.; Joung, J.; Verdine, V.; Donghia, N.; Daringer, N. M.; Freije, C. A.; Myhrvold, C.; Bhattacharyya, R. P.; Livny, J.; Regev, A.; Koonin, E. V.; Hung, D. T.; Sabeti, P. C.; Collins, J. J.; Zhang, F. *Science* **2017**, *356*, 438–442.

(8) Chen, J. S.; Ma, E.; Harrington, L. B.; Da Costa, M.; Tian, X.; Palefsky, J. M.; Doudna, J. A. *Science* **2018**, *360*, 436–439.

(9) Li, S.-Y.; Cheng, Q.-X.; Liu, J.-K.; Nie, X.-Q.; Zhao, G.-P.; Wang, J. *Cell Res.* **2018**, *28*, 491–493.

(10) Zhu, C. S.; Liu, C. Y.; Qiu, X. Y.; Xie, S. S.; Li, W. Y.; Zhu, L.; Zhu, L. Y. *Biotechnol. Bioeng.* **2020**, *117*, 2279–2294.

(11) Bhattacharyya, R. P.; Thakku, S. G.; Hung, D. T. *ACS Infect. Dis.* **2018**, *4*, 1278–1282.

(12) Lindahl, T. *Proc. Natl. Acad. Sci. U.S.A.* **1974**, *71*, 3649–3653.

(13) Longo, M. C.; Berninger, M. S.; Hartley, J. L. *Gene* **1990**, *93*, 125–128.

(14) Hsieh, K.; Mage, P. L.; Csordas, A. T.; Eisenstein, M.; Soh, H. T. *Chem. Commun.* **2014**, *50*, 3747–3749.

(15) Zhou, R.; Li, Y.; Dong, T.; Tang, Y.; Li, F. *Chem. Commun.* **2020**, *56*, 3536–3538.

(16) Wang, R.; Qian, C.; Pang, Y.; Li, M.; Yang, Y.; Ma, H.; Zhao, M.; Qian, F.; Yu, H.; Liu, Z.; Ni, T.; Zheng, Y.; Wang, Y. *Biosens. Bioelectron.* **2021**, *172*, 112766.

(17) Subbarao, K.; Mahanty, S. *Immunity* **2020**, *52*, 905–909.

(18) Webster, R. G.; Govorkova, E. A. *Ann. N. Y. Acad. Sci.* **2014**, *1323*, 115–139.

(19) Borchers, A. T.; Chang, C.; Gershwin, M. E.; Gershwin, L. J. *Clin. Rev. Allergy Immunol.* **2013**, *45*, 331–379.

(20) Galloway, S. E.; Paul, P.; MacCannell, D. R.; Johansson, M. A.; Brooks, J. T.; MacNeil, A.; Slayton, R. B.; Tong, S.; Silk, B. J.; Armstrong, G. L.; Biggerstaff, M.; Dugan, V. G. *Morb. Mortal. Wkly. Rep.* **2021**, *70*, 95–99.

(21) Durner, J.; Burggraf, S.; Czibere, L.; Tehrani, A.; Watts, D. C.; Becker, M. *Dent. Mater.* **2021**, *37*, e95–e97.

(22) Medrano, R. F. V.; de Oliveira, C. A. *Mol. Biotechnol.* **2014**, *56*, 599–608.

(23) Zhang, C.; Yao, Y.; Zhu, J.-L.; Zhang, S.-N.; Zhang, S.-S.; Wei, H.; Hui, W.-L.; Cui, Y.-L. *Sci. Rep.* **2016**, *6*, 26533.

(24) Malpartida-Cardenas, K.; Rodriguez-Manzano, J.; Yu, L.-S.; Delves, M. J.; Nguon, C.; Chotivanich, K.; Baum, J.; Georgiou, P. *Anal. Chem.* **2018**, *90*, 11972–11980.

(25) Fuchs, R. T.; Curcuro, J.; Mabuchi, M.; Yourik, P.; Robb, G. B. Cas12a trans-cleavage can be modulated in vitro and is active on ssDNA, dsDNA, and RNA. **2019**, bioRxiv:600890.

(26) Zuker, M. *Nucleic Acids Res.* **2003**, *31*, 3406–3415.

(27) Li, S.-Y.; Cheng, Q.-X.; Wang, J.-M.; Li, X.-Y.; Zhang, Z.-L.; Gao, S.; Cao, R.-B.; Zhao, G.-P.; Wang, J. *Cell Discovery* **2018**, *4*, 20.

(28) Bae, S.; Park, J.; Kim, J.-S. *Bioinformatics* **2014**, *30*, 1473–1475.

(29) Haussler, M.; Schönig, K.; Eckert, H.; Eschstruth, A.; Mianné, J.; Renaud, J.-B.; Schneider-Maunoury, S.; Shkumatava, A.; Teboul, L.; Kent, J.; Joly, J.-S.; Concordet, J.-P. *Genome Biol.* **2016**, *17*, 148.

(30) Labun, K.; Montague, T. G.; Krause, M.; Torres Cleuren, Y. N.; Tjeldnes, H.; Valen, E. *Nucleic Acids Res.* **2019**, *47*, W171–W174.

(31) Wang, B.; Wang, R.; Wang, D.; Wu, J.; Li, J.; Wang, J.; Liu, H.; Wang, Y. *Anal. Chem.* **2019**, *91*, 12156–12161.

(32) Wu, H.; Qian, C.; Wu, C.; Wang, Z.; Wang, D.; Ye, Z.; Ping, J.; Wu, J.; Ji, F. *Biosens. Bioelectron.* **2020**, *157*, 112153.

- (33) Wu, H.; He, J.-S.; Zhang, F.; Ping, J.; Wu, J. *Anal. Chim. Acta* **2020**, *1096*, 130–137.
- (34) Wang, R.; Chen, R.; Qian, C.; Pang, Y.; Wu, J.; Li, F. *Sens. Actuators, B* **2021**, *326*, 128618.
- (35) Ooi, K. H.; Liu, M. M.; Tay, J. W. D.; Teo, S. Y.; Kaewsapsak, P.; Jin, S.; Lee, C. K.; Hou, J.; Maurer-Stroh, S.; Lin, W.; Yan, B.; Yan, G.; Gao, Y.-G.; Tan, M. H. *Nat. Commun.* **2021**, *12*, 1739.
- (36) Kox, L. F.; Rhienthong, D.; Miranda, A. M.; Udomsantisuk, N.; Ellis, K.; van Leeuwen, J.; van Heusden, S.; Kuijper, S.; Kolk, A. H. *J. Clin. Microbiol.* **1994**, *32*, 672–678.
- (37) Fonfara, I.; Richter, H.; Bratovič, M.; Le Rhun, A.; Charpentier, E. *Nature* **2016**, *532*, 517–521.
- (38) Zetsche, B.; Gootenberg, J. S.; Abudayyeh, O. O.; Slaymaker, I. M.; Makarova, K. S.; Essletzbichler, P.; Volz, S. E.; Joung, J.; van der Oost, J.; Regev, A.; Koonin, E. V.; Zhang, F. *Cell* **2015**, *163*, 759–771.
- (39) Tanner, N. A.; Evans, T. C. *Curr. Protoc. Mol. Biol.* **2014**, *105*, 15.14.1–15.14.14.
- (40) Lee, D.; Shin, Y.; Chung, S.; Hwang, K. S.; Yoon, D. S.; Lee, J. H. *Anal. Chem.* **2016**, *88*, 12272–12278.
- (41) Qian, C.; Wang, R.; Wu, H.; Zhang, F.; Wu, J.; Wang, L. *Anal. Chem.* **2019**, *91*, 11362–11366.
- (42) Badolo, A.; Okado, K.; Guelbeogo, W. M.; Aonuma, H.; Bando, H.; Fukumoto, S.; Sagnon, N. F.; Kanuka, H. *Malar. J.* **2012**, *11*, 227.
- (43) Li, L.; Li, S.; Wu, N.; Wu, J.; Wang, G.; Zhao, G.; Wang, J. *ACS Synth. Biol.* **2019**, *8*, 2228–2237.