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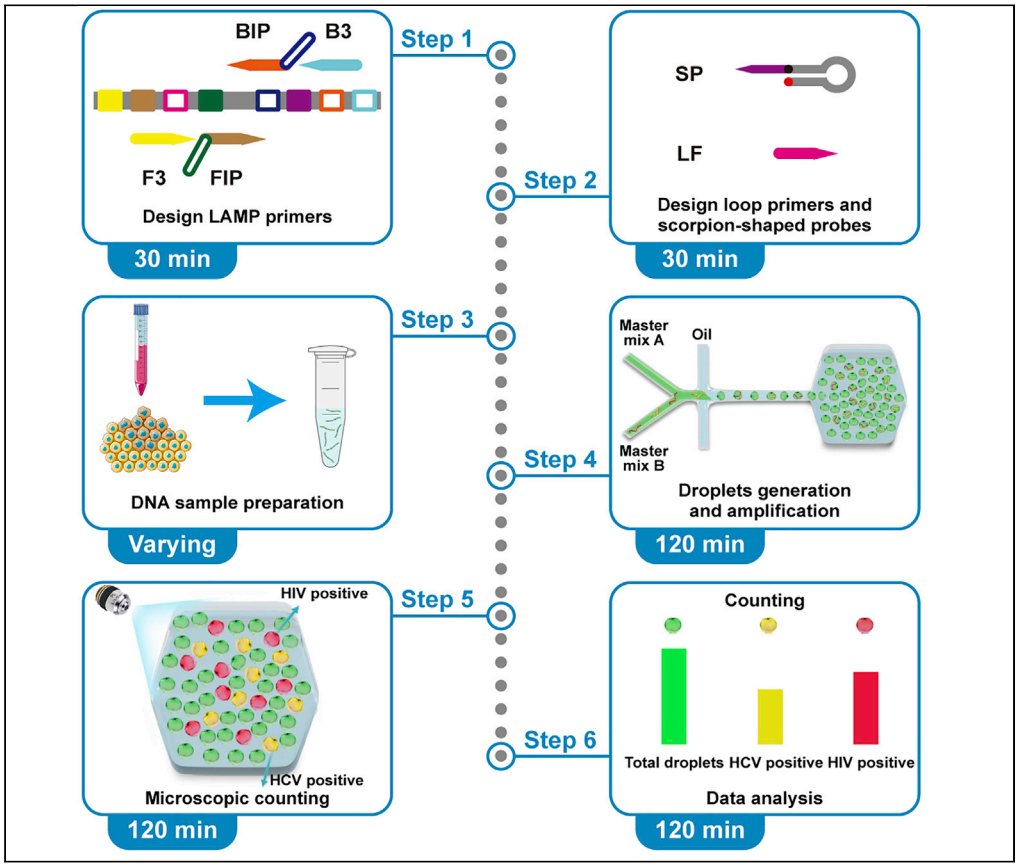
Protocol

Droplet microfluidic-based loop-mediated isothermal amplification (dLAMP) for simultaneous quantification of multiple targets



The quantification of trace nucleic acids in biological samples is a frequent requirement in experimental and clinical diagnostics. Here, we present a protocol for the digital quantification of multiple nucleic acid targets with droplet microfluidics-based loop-mediated isothermal amplification (dLAMP). Our protocol provides a fundamental platform for the absolute quantification of multiple nucleic acid targets with high specificity, allowing readily adaption in various diagnostic settings.

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Highlights

Protocol for droplet microfluidic-based loop-mediated isothermal amplification (dLAMP)

Fluorescence-activating scorpion-shaped probes-based dLAMP for fluorescence generation

Fast and accurate fluorescence microscopy-based droplets counting

Can be applied for the absolute quantification of multiple nucleic acid targets

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Droplet microfluidic-based loop-mediated isothermal amplification (dLAMP) for simultaneous quantification of multiple targets

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SUMMARY

The quantification of trace nucleic acids in biological samples is a frequent requirement in experimental and clinical diagnostics. Here, we present a protocol for the digital quantification of multiple nucleic acid targets with droplet microfluidics-based loop-mediated isothermal amplification (dLAMP). Our protocol provides a fundamental platform for the absolute quantification of multiple nucleic acid targets with high specificity, allowing readily adaption in various *in vitro* diagnostic settings. For complete details on the use and execution of this protocol, please refer to Tan et al. (2021a, 2021b).

BEFORE YOU BEGIN

LAMP is advantageous for its high efficiency, potentially amplifying the target template into 10^9 copies within a one-step isothermal reaction (Nagamine et al., 2002; Notomi et al., 2000). dLAMP has been reported for quantifying the presence of ultralow amounts of sequences characterized for infectious diseases and early-stage cancers (Hu et al., 2017; Kreutz et al., 2019; Schoepp et al., 2017; Yuan et al., 2018). dLAMP is mainly based on micro-reaction chamber arrays, bead encapsulation emulsion, or droplet focusing microfluidic chips (Chen et al., 2018; Hu et al., 2020; Xia et al., 2017; Yu et al., 2019, 2020).

The protocol below describes a multiplexed dLAMP technique for simultaneous quantification of multiple nucleic acid target sequences using fluorescence-activating scorpion-shaped probes (SPs), microfluidic-assisted droplet generation and fluorescence microscopy-based counting. This technology has been successfully applied for the digital detection of HCV and HIV RNA in plasma samples, and the absolute quantification of DAPK1 gene methylation in cervical cancer patients, providing a fundamental platform for developing affordable, high-throughput, and digital nucleic acid diagnosis methods for various diagnostic nucleic acid targets.

Institutional permissions

Plasma samples were collected by the Third Xiangya Hospital of Central South University with informed consent and with the approval from the ethical committee of the Third Xiangya Hospital of Central South University.

Design LAMP primers and scorpion-shaped probes

Timing: 1–2 weeks to receive primers and probes

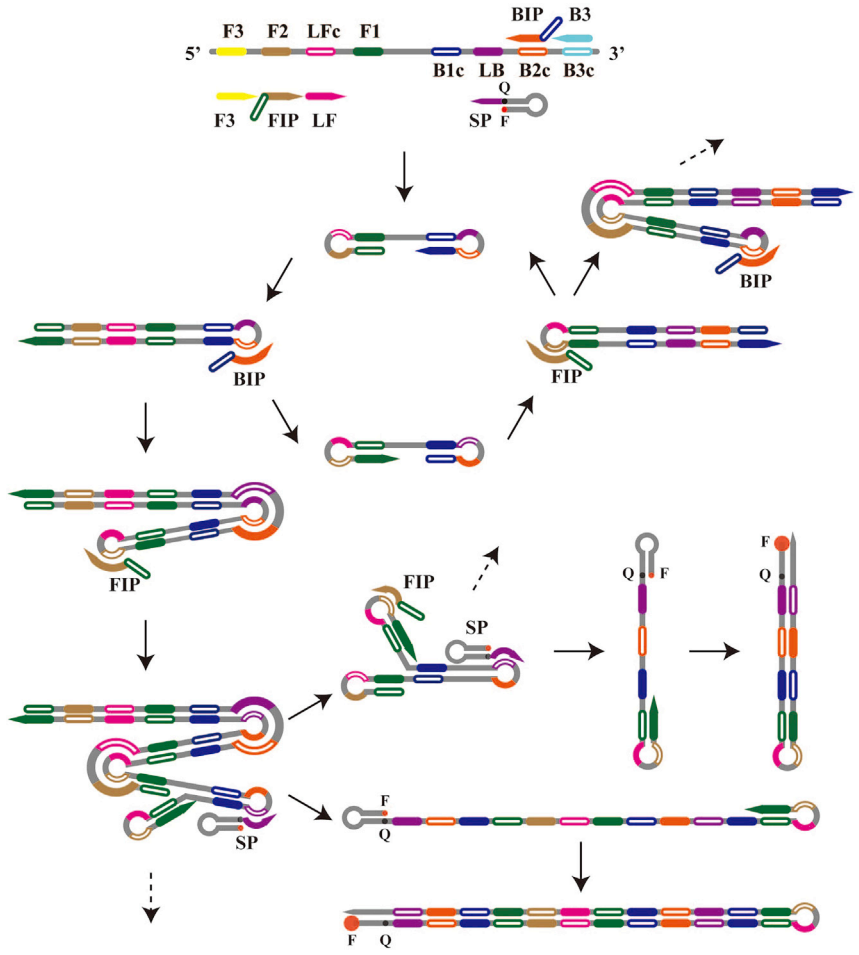

Figure 1. Illustration of SP-based LAMP reaction

Eight distinct regions are designated on the target DNA, labeled F3, F2, LFc, F1, B1c, LB, B2c, and B3 from the 5′ end ('c' represents a complementary region, hollow regions are complemented to the fulfilled regions of the same colors). Inner primers FIP/BIP consist of the F1c/B1c sequence and the F2/B2 sequence. F3/B3, LF, SP stand for outer primers, loop forward primer, and scorpion-shaped probe, respectively. FIP/BIP: Initiating the replicative DNA synthesis by the *Bst* DNA polymerase, extending and assisting the template destruction, playing a major role in the cycling amplification step. F3/B3: Displacing the newly synthesized DNA strand by FIP/BIP and releasing the target DNA. LF: Hybridizing the stem-loops and accelerating the LAMP reaction. SP: Generating fluorescence signal and accelerating the LAMP reaction.

The LAMP technology typically uses two sets of primers targeting six regions of the DNA sequence and a strand-displacing *Bst* DNA polymerase. LAMP involves three critical steps: 1) the formation of the dumbbell-liked single-stranded loop structure, 2) the generation of multimeric DNAs consisting of inverted repeats by cycling amplification, 3) the acceleration of amplification by loop primers (Nagamine et al., 2002; Notomi et al., 2000; Tomita et al., 2008). In the multiplexed dLMAP method reported in this protocol, SPs were used to facilitate the in-situ activation of fluorescence. The basic principle of SP-based LAMP is illustrated in Figure 1. The design of LAMP primers is as follow:

1. Use LAMP primer design software PrimerExplorer V5 (http://primerexplorer.jp/e/index.html) to design LAMP primers. Refer to the corresponding guide manual for detailed instructions (http://primerexplorer.jp/e/v4_manual/).

Key factors to be considered in primer design:

a. For GC-rich sequences, the T_m value of primer should be controlled at 60°C–65°C, 55°C–60°C for AT-rich sequences.

b. The length of F2, F1, B1c, and B2c should be 18–24 nucleotides (nt), and the length of F3, B3c, LF, and LB regions should be 17–21 nt.
c. The length of the amplification region (between F2 to B2) should be 120–180 base pairs (bp). The distance between F2 and F3 (or B2 and B3) should be 0–20 bp. The distance between F2 and F1 (or B2 and B1) should be 40–60 bp.
d. The Gibbs free energy of F2/B2, F3/B3, LF/LB, and F1c/B1c should be less than -4 kcal/mol.
e. The designed primers should avoid the formation of secondary structures.
f. If the target sequence has a restriction enzyme cleavage site besides the primer region, this site could be used for the identification of amplification products.

2. Use PrimerExplorer to design the loop primers and SPs.
   a. Follow the software instruction to design the loop primers, then use one of them to design the SP.
   b. Design a hairpin structure with a quencher (BHQ2) and a fluorophore linked at the two ends of the strand.
   c. The SP consists of a loop primer and a hairpin structure. We typically linked the 5′-end of the loop primer to the 3′-end of the hair structure to obtain the SP.

   Key factors to be considered in loop primer and SPs design:
      i. The design of the hairpin structure needs to follow the basic design principles of a molecular beacon.
      ii. Optimize the base pair (bp) number and sequence within the stem region of the molecular beacon to ensure optimal reaction efficiency. The typical bp number is 5–7, GC% is 75%–100%.
      iii. Whether one or multiple targets, avoid any unwanted hybridization between all designed LAMP primers and SPs.

Note: SP acts as a loop primer to amplify the target sequence and offers real-time fluorescence monitoring of the LAMP reaction.

DNA template preparation from clinical specimens

Timing: Varied, depends on the type and number of samples

Timing: 5 h for 20 samples for step 3.a

Timing: 120 min for 20 samples for step 3.b

3. All nucleic acid targets need to be in or converted to single-stranded or double-stranded DNA form. All clinical specimens need to be obtained ethically legal.
   a. For virus RNA extraction from blood samples, inactivate patient samples and extract RNA via the QIAamp MinElute Virus Spin Kit per manufacturer's protocol. The process is strictly performed in Biosafety Level 2 laboratory. For the reverse transcription, please refer to the instruction of a commercially available kit, for example, the QuantiTect Reverse Transcription Kit.
   b. For genomic DNA extraction from tissue samples, use the Ezup Column FFPE DNA purification kit to extract the genomic DNA.
   c. For other nucleic acid target extraction, please refer to corresponding protocols.

Note: We recommend using reliable and commonly used protocols for DNA sample preparation. Moreover, it is crucial to have a quality control process during the DNA sample preparation process. Gel electrophoresis and PCR are recommended.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human plasma samples (male and female, age 20–60)	The Third Xiangya Hospital of Central South University	N/A
Chemicals, peptides, and recombinant proteins		
TE buffer (pH 8.0)	Sangon Biotech	Cat. #B548106-0500
Tris-borate-EDTA (TBE) buffer	Sangon Biotech	Cat. #B548102-0500
DNA Marker (100–5,000 bp)	Sangon Biotech	Cat. #B500351-0500
Bovine serum albumin (BSA)	Sangon Biotech	Cat. #C500642-0001
RNase-free water	Sangon Biotech	Cat. #B541018-0010
Agarose	BBI Life Sciences	Cat. #A600014
Goldview	Beijing Dingguo Changsheng Biotechnology	Cat. #DH392-5
Ethidium bromide	Beijing Dingguo Changsheng Biotechnology	Cat. #GE117-1G
Bst 2.0 WarmStart™ DNA polymerase	New England Biolabs	Cat. #M0537L
DNase I (RNase-Free)	New England Biolabs	Cat. #M0303S
Thermopol buffer	New England Biolabs	Cat. #B9004S
Deoxyribonucleotides (dNTPs) mixture	New England Biolabs	Cat. #N0447L
5% Pico-Surf™ 1 in Novec-7500	Dolomite Microfluidics	Cat. #3200216
HFE-7500	Dolomite Microfluidics	Cat. #3200570
Fluorescein sodium	Sigma-Aldrich	Cat. #1.03887
$MgSO_4$	Sigma-Aldrich	Cat. #M2643
Octadecyltrichlorosilane	Sigma-Aldrich	Cat. #104817
Critical commercial assays		
QIAamp MinElute Virus Spin Kit	QIAGEN	Cat. #57704
QuantiTect Reverse Transcription Kit	QIAGEN	Cat. #205311
Ezup Column FFPE DNA purification kit	Sangon Biotech	Cat. #B518269
Oligonucleotides		
mLAMP primers and probes (See Table 1)	Sangon Biotech	N/A
HCV and HIV plasmid with pUC57 vector	GenScript	N/A
Software and algorithms		
Prism 8.0	GraphPad	https://www.graphpad.com/
Origin 2018	OriginLab	https://www.originlab.com/
Adobe Illustrator 2020	Adobe	https://www.adobe.com/cn/
AutoCAD 2014	Autodesk	https://www.autodesk.com.cn/
NIS-Element	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
PrimerExplorer V5	PrimerExplorer	http://primerexplorer.jp/e/index.html
Blastn suite	BLAST	https://blast.ncbi.nlm.nih.gov/Blast.cgi
Other		
C1000 Thermal Cycler	Bio-Rad	Cat. #1841100
Benchtop centrifuge	Sangon Biotech	Cat. #G508010-0001
Tanon 4200SF Gel Imaging System	Tanon Science & Technology	N/A
ABI StepOnePlus qPCR instrument	Applied Biosystems	Cat. #4376600
TS-2A Syringe Pump System	Longer Precision Pump	Cat. #05.03.16A
Nikon TI-E+A1 SI confocal laser scanning microscope	Nikon	N/A

Table 1. Synthesized DNA primers and templates for multiplexed dLAMP analysis

Name	Sequence (5′ to 3′)
HCV-F3	TGGTCTGCGGAACCGG
HCV-B3	GGGGCACTCGCAAGCA
HCV-FIP	ACGCCCAAATCTCCAGGCATTGCATTGCCAGGACGACCGG
HCV-BIP	CCGCGAGACTGCTAGCCGACCCTATCAGGCAGTA
HCV-LF	AGCGGGTTGATCCAAGAAAGGAC
HCV-LB	TGTTGGGTCGCGAAAGGCC
HCV-SP	TAMRA-AGCGCGGATATCTCACCGCGCT(BHQ2)TGTT GGGTCGCGAAAGGCC
HCV template	GCCAGCCCCCTGATGGGGGCGACACTCCACCATGAATCACTCC CCTGTGAGGAACTACTGTCTTCACGCAGAAAGCGTCTAGCCATG GCGTTAGTATGAGTGTCGTGCAGCCTCCAGGACCCCCCCTCCCG GGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAAT TGCCAGGACGACCGGGTCCTTTCTTGGATCAACCCGCTCAATGC CTGGAGATTTGGGCGTGCCCCCGCGAGACTGCTAGCCGAGTAG TGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGGGTGCT TGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGC ACCATGAGCACGAATCCTAAAC
HIV-F3	CCTATTTGTTCCTGAAGGGT
HIV-B3	ATTATCAGAAGGAGCCACC
HIV-FIP	GAGTGCATCCAGTGCATGCACTGCTATGTCACTTCCCCT
HIV-BIP	CCATTCTGCAGCTTCCTCATTGAACACCATGCTAAACACAGT
HIV-LF	CAGGCCAGATGAGAGAACCA
HIV-LB	ATGGCTGCTTGATGTCCCC
HIV-SP	Cy5-AGCGCGGATATCTCACCGCGCT(BHQ2)ATGGCTG CTTGATGTCCCC
HIV template	ATTTTATTTAATCCCAGGATTATCCATCTTTTATAAATTTCTCCTACT GGGATAGGTGGATTATTTGTCATCCATCCTATTTGTTCCTGAAGG GTACTAGTAGTTCCTGCTATGTCACTTCCCCTTGGTTCTCTCATCT GGCCTGGTGCAATAGGCCCTGCATGCACTGGATGCACTCTATCC CATTCTGCAGCTTCCTCATTGATGGTCTCTTTTAACATTTGCATGG CTGCTTGATGTCCCCCCACTGTGTTTAGCATGGTGTTTAAATCTTG TGGGGTGGCTCCTTCTGATAATGCTGAAAACATGGGTATCACTTC TGGGCTGAAAGCCTTCTCTTCTACTACTTTTACCCATGCATTT AAAGTTCTAGGTGATATGGCCTGATGTACCA
HCV forward primer	CACTCGCAAGCACCCTATCA
HCV reverse primer	AGCCATAGTGGTCTGCGGA
HIV forward primer	GTAGTTCCTGCTATGTCACTTCCC
HIV reverse primer	CATTATCAGAAGGAGCCACCC

STEP-BY-STEP METHOD DETAILS

Microfluidic chip fabrication

Timing: 24 h

Fabricate microfluidic chips. In this protocol, glass microchips are used for their good reusability. Microchips made from other materials, including polycarbonate, polymethylmethacrylate, polydimethylsiloxane can potentially be used too.

1. Designing mask. Use AutoCAD or similar software to draw the Y-shape droplet microfluidic chip and droplet counting microwell chip pattern (for the use of AutoCAD, please refer to http://www.cadtutor.net/tutorials/autocad/index.php), order the photolithography mask with the designed pattern from an available commercial supplier (Figure 2, Data S1 and S2).
2. Fabricating microfluidic chips. The fabrication of the designed microfluidic chips followed well-developed and described protocols (Golozar et al., 2020; Jia et al., 2004). Overall, it includes photoresist developing, mask exposing, selective chromium removal, glass etching, and chip bonding (troubleshooting 1).

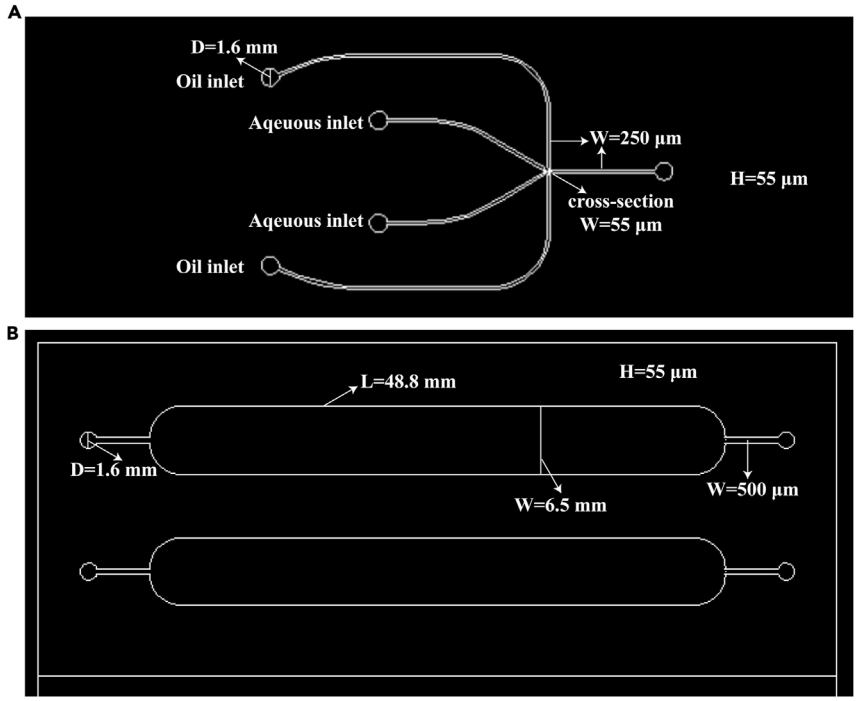

Figure 2. Mask design of microfluidic chips

(A) The Y-shape droplet microfluidic chip (Notes: The two oil inlets can be merged into one).

(B) The droplet counting microwell chip.

Note: It is critical to remove any bubbles that may form when bonding the chip.

3. Hydrophobic treatment. Infiltrate the Y-shape droplet microfluidic chip and droplet counting microwell chip channel with 0.1% (v/v) perfluorododecyltrichlorosilane (FTCS) solution in dry toluene for 5 min (troubleshooting 2).

⚠ CRITICAL: Wearing appropriate protective clothing and equipment when handling highly toxic and moisture-sensitive FTCS solution and use only in a fume hood.

Alternatives: The hydrophobic modification on the glass substrate microfluidic chip can be carried out by plasma technology.

4. Washing. Flush the microfluidic channels with pure HFE-7500 fluorinated oil to remove unreacted silanes and flush each module with pressurized air or nitrogen. The chips are ready for experimental use.

⚠ CRITICAL: The chip can be stored for weeks or months at 20°C–25°C before use. To prevent degradation of the treated surface, avoid direct sunlight. The chip surface should be covered with Scottish tape to prevent dust.

Standard multiplexed LAMP reaction

Timing: 120 min

The multiplexed LAMP reaction procedure is demonstrated by simultaneously detecting HCV and HIV plasmids, in which HCV and HIV templates were cloned into empty pUC57 vectors, and an appropriate primer design is shown in Table 1.

Note: When designing the primers for LAMP assays, perform specificity analysis with BLAST to find the specific sequences for the multiple targets of interest.

5. HCV and HIV plasmids are heated to 95°C for 5 min and then cooled with an ice/water mixture for at least 20 min before use.

Note: Such quenching can open DNA double helix structure, resulting in better hybridization with primers.

6. Prepare the master mix for LAMP reaction in a microcentrifuge tube.

LAMP master mix per reaction

Reagent	Final concentration	Amount
ThermoPol buffer (10×)	1×	2.5 μL
HCV-FIP (40 μM)	1.6 μM	1 μL
HCV-BIP (40 μM)	1.6 μM	1 μL
HCV-F3 (5 μM)	0.2 μM	1 μL
HCV-B3 (5 μM)	0.2 μM	1 μL
HCV-LF (10 μM)	0.4 μM	1 μL
HCV-SP (10 μM)	0.4 μM	1 μL
HIV-FIP (40 μM)	1.6 μM	1 μL
HIV-BIP (40 μM)	1.6 μM	1 μL
HIV-F3 (5 μM)	0.2 μM	1 μL
HIV-B3 (5 μM)	0.2 μM	1 μL
HIV-LF (10 μM)	0.4 μM	1 μL
HIV-SP (10 μM)	0.4 μM	1 μL
$MgSO_4$ (100 mM)	4 mM	1 μL
dNTPs (10 mM)	1.6 mM	4 μL
Bst 2.0 WarmStart™ DNA polymerase (8 U/μL)	0.32 U/μL	1 μL
ddH_2O	N/A	2.5 μL
Total	**N/A**	**23 μL**

Prepare freshly before use.

Note: Due to volume loss during pipetting, a master mix solution allowing for 2–3 additional reactions is suggested.

⚠ CRITICAL: Preparing and maintaining the master mix on ice boxes is recommended to avoid enzyme activity decreasing and nonspecific product amplification.

7. Add 1 μL HCV plasmid, 1 μL HIV plasmid, and 23 μL LAMP master mix to a respective PCR 8-Tube strip and gently mix by pipetting 7–10 times.

Note: A no template control (NTC) should be used (nuclease-free H_2O only as sample) to indicate false-positive results due to contamination of reagents or work area (troubleshooting 3).

⚠ CRITICAL: Be careful not to form bubbles while mixing.

8. Spin PCR 8-Tube Strip tubes lightly in a Benchtop centrifuge at 2,000 × *g* for roughly 10 s.
9. Run the LAMP reaction at a set temperature using a C1000 Thermal Cycler with a CFX96 detection system. Monitor the real-time fluorescence intensity in ROX and Cy5 channels at intervals of 30 s for 100 cycles (troubleshooting 4).

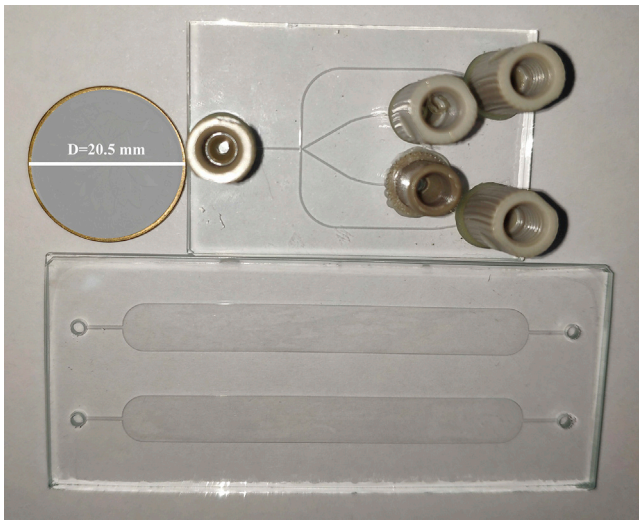

Figure 3. Image of droplet generation (top) and counting microwell (bottom) chips

Note: Firstly, when developing a new multiplexed LAMP assay, optimize the LAMP reaction temperature by performing parallel experiments at different temperatures (the optimized temperature gives the minimum Ct value). For this assay, the optimized reaction temperature is 64°C.

Secondly, ROX channel and Cy5 channel indicate HCV and HIV in the experiment, respectively. Combinations of other fluorophores are also feasible (Ball et al., 2016; Becherer et al., 2018; Jiang et al., 2015; Varona and Anderson, 2019). For more combinations, use the PrimeTime Multiplex Dye Selection Tool from Integrated DNA Technologies. In addition, make sure the selected combinations are compatible with the fluorescence microscope to be used.

Gel electrophoresis of LAMP products

Timing: 120 min

To further confirm the success of the LAMP reaction, gel electrophoresis is recommended.

10. Heat the reaction products to 80°C for 20 min to stop the reaction by denaturing the polymerase.

Note: After the run is completed, reaction products can be stored at −20°C for up to several weeks.

11. Analyze the LAMP products with agarose gel electrophoresis. 3% agarose gel is used, gel electrophoresis is carried out in 0.5 × TBE electrophoresis buffer, gels are stained with 0.5 μg/mL ethidium bromide and 0.5 μg/mL Goldview. Gels are visualized with Tanon 4200SF gel imaging system (Figure 3).

⚠ CRITICAL: Firstly, be careful when using ethidium bromide. Gloves should be worn during preparation and use. Secondly, gel electrophoresis is a common cause of contamination. It is recommended to perform this experiment in an independent lab.

Alternatives: Other gel stains can also be used (e.g., 4S GelRed, Sangon Biotech, Cat #A616697-0100; SYBR™ Safe DNA Gel Stain, Invitrogen™, Cat #S33102).

Multiplexed dLAMP reaction

Timing: 120 min

Apply the multiplexed dLAMP platform to detect HCV and HIV.

12. Clean the microfluidic chips with Kimwipes and ethanol to remove dust and particles.

Note: The dLAMP experiment is best carried out in a dust-free laboratory to avoid any potential chip contamination and for the purpose of chip reuses.

13. Prepare two sterile 1-mL syringes to inject continuous phase consisting of fluorinated oil Novec-7500 containing 1% (wt/wt) Pico-Surf™ fluorinated surfactant.

⚠ CRITICAL: Filtration of the prepared oil phase with polyethersulfone membranes or cellulose acetate membranes with a membrane pore size of 0.1/0.2 μm is recommended.

Note: The surfactant acts as an interface stabilization agent that stabilizes the droplets under a wide range of temperature and biological conditions, as well as allowing droplets to be stored for several weeks in suitable conditions. The optimal surfactant concentration needs to be determined to allow the stable formation of droplets during the whole experiment (Baret, 2012).

14. Prepare the multiplexed dLAMP reaction master mix A in a microcentrifuge tube.

Multiplexed dLAMP reaction master mix A

Reagent	Final concentration	Amount
ThermoPol buffer (10×)	2×	5 μL
HCV target	different concentration	1 μL
HIV target	different concentration	1 μL
ddH_2O	N/A	18 μL
Total	N/A	**25 μL**

Store at 4°C for up to 1 week.

Multiplexed dLAMP reaction master mix B

Reagent	Final concentration	Amount
HCV-FIP (80 μM)	3.2 μM	1 μL
HCV-BIP (80 μM)	3.2 μM	1 μL
HCV-F3 (10 μM)	0.4 μM	1 μL
HCV-B3 (10 μM)	0.4 μM	1 μL
HCV-LF (20 μM)	0.8 μM	1 μL
HCV-SP (20 μM)	0.8 μM	1 μL
HIV-FIP (80 μM)	3.2 μM	1 μL
HIV-BIP (80 μM)	3.2 μM	1 μL
HIV-F3 (10 μM)	0.4 μM	1 μL
HIV-B3 (10 μM)	0.4 μM	1 μL
HIV-LF (20 μM)	0.8 μM	1 μL
HIV-SP (20 μM)	0.8 μM	1 μL
$MgSO_4$ (200 mM)	8 mM	1 μL
dNTPs (10 mM)	1.6 mM	8 μL
Bst 2.0 WarmStart™ DNA polymerase (8 U/μL)	0.32 U/μL	2 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Fluorescein sodium (25 μM)	1 μM	1 μL
ddH_2O	N/A	1 μL
Total	N/A	**25 μL**

Prepare freshly before use.

15. Prepare the multiplexed dLAMP reaction master mix B in another microcentrifuge tube.

    Note: Optionally, BSA (0.2 mg/mL) can be added to the reaction solution to lessen the enzyme loss during pipetting and transferring, and to stabilize the enzyme in reaction.

16. Use two 1-mL disposable sterile syringes (26 gauge) to inject dLAMP reaction master mix A and B, separately.

    Note: The syringes need to match the PEEK adapters.

17. Connect the syringes to the corresponding entrances of the Y-shape droplet microfluidic chip through PTFE microtubes (1/16 in. OD × 0.01 in. ID), and PEEK adapters (1/16 in. OD). The injection flow rates are set to 10 μL/min for oil phases and 3 μL/min for aqueous phases during droplet generation.

    Note: The injection flow rates of oil and water phases are determined according to the structure of the chip.

18. Connect the droplet generation chip exit to the droplet counting microwell chip with a PTFE microtube, and PEEK adapters to collect the generated droplets (Figure 4).

    Note: Linear connectors can be used to connect and fix the chip to minimize the experimental vibration, as it provides a straight-through flow path from the chip to the tube, which is beneficial for droplet collection.

19. Observe the flow junction of the droplet generation chip under a microscope with a 10 × objective. Purge off air from the chip with buffer solution, wait until droplet generation stabilizes, then collect the droplets (troubleshooting 5).
20. Observe the collection chip to monitor the droplet generation process. Droplets should be round with uniform diameters (~50 μm) and monodispersed in the chamber (troubleshooting 6).

    Note: Ensure that sufficient oil exists below the emulsion to prevent droplet evaporation and drying (troubleshooting 7).

21. When the droplet counting microwell chip is full of monodisperse droplets, detach it from the droplet generation chip, and connect a new droplet counting microwell chip to the droplet generation chip exit to start collecting droplets for another sample.

    Note: The collection chamber can collect about 61,000 droplets, the active collection time is about 40 s. To avoid sample cross-interference, use a new injector when injecting a different sample and discard the droplets generated in the first two minutes. This process also allows the droplet generation of the new sample to be stabilized. The typical collection time for one sample is about 3 min. After collecting the droplets, place the droplet counting microwell chips in a thermostat water bath (64°C) for 60 min to allow the LAMP reaction to occur.

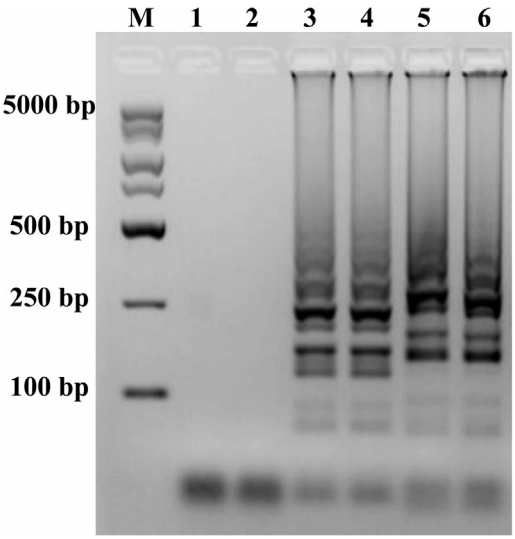

Figure 4. Gel images of LAMP reaction products
Lane M: Marker; lane 1: blank; lane 2:HeLa cell genomic DNA; lane 3: HCV template; lane 4: cDNA from HCV-infected sample; lane 5: HIV template; lane 6: cDNA from HIV-infected sample.

⚠ CRITICAL: To prevent the loss of droplets and avoid water entering the chip during water bath incubation, use tape to seal the droplet counting microwell chips.

Note: Optimize the dLAMP reaction time to allow the stabilization of fluorescent droplet generation. For this assay, the optimized reaction time is 60 min.

22. After the LAMP reaction, take out the droplet counting microwell chips, wipe the water on the surface off the chip, and analyze them with microscopic imaging.

Droplet counting by fluorescence imaging

Timing: 120 min

Acquire fluorescence images of the entire microwells using an inverted confocal laser scanning microscope under 10× objective.

23. Preparing. Place the droplet counting microwell chip on the microscope counter. Open the imaging software and select appropriate scan modes.

Note: Nikon Inverted Eclipse Ti epifluorescence microscope equipped with a motorized XY stage (Nikon), a CoolLed pE-4000 illumination source, a Nikon DS-Qi2 camera, and an apochromatic 10× objective (numerical aperture, 0.45) (Nikon) is used here.

24. Focusing. Adjust the microscope so that the droplets can be clearly observed through eyepieces.

Note: The average droplet diameter is around 50 μm. 10× objective is recommended for observation and image scanning.

25. Setting up. Setup image acquisition parameters for 488 (FITC), 560 (TAMRA), and 640 (Cy5) channels. Optimize imaging parameters until the positive droplets are brightly fluoresced under observation (troubleshooting 8). Save the parameters for later use (Figure 5).
26. Imaging. To image all droplets, use the large image scan function. The microscope will automatically scan the defined region and combine them into a large image (Figure 6). The scan parameters are as follows: pixel size is 2.49 μm, optical resolution is 0.78 μm, scan size is 512 × 512 pixels, and dwell time is 2.2 μs.

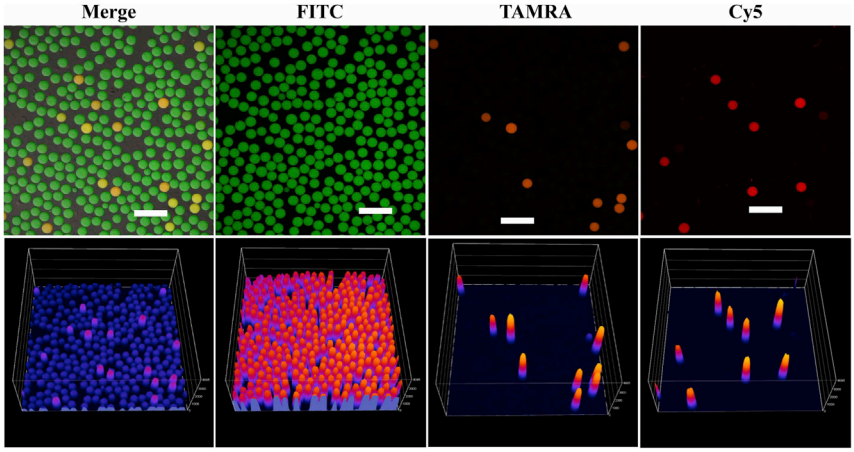

Figure 5. Fluorescence images and intensity profiles of droplets in different channels
Scale bar: 200 μm.

Note: Set the scan area according to the size of the microwell. It takes about 10 min to scan for one large image. In addition, the scanning time depends on the selected scanning parameters of the microscope.

27. Saving. Save the scanned large image and image other samples under the same microscopy parameters (troubleshooting 9).
28. Cleaning. Clean the chips after all the sample scans are finished. To reuse the microchips, inject isopropanol to clean the chips, then incubate with 2 U/μL DNase I at 37°C for 8–12 h to degrade residual oligonucleotides.

Note: Clean the droplet counting microwell chip and Y-shape droplet microfluidic chip in a fume hood to avoid aerosol pollution.

Droplet analysis

Timing: 120 min

Analyze the pictures and count positive droplets with NIS-Element software (or other image analysis software, i.e., ImageJ), calculate the template copies according to the Poisson distribution.

29. Open the images in NIS-Element software.

30. Click tool bar, choose View > Analysis Controls > Automated Measurement > Thresholding > Per channel. Properly define the values of fluorescence intensity threshold, droplet size ranges, circularity and so on. After automated analysis, positive droplets are selected and counted (Figure 7).

Proper parameter settings are critical for droplet counting results.

a. For the fluorescence intensity threshold, set the threshold for 488 channel above the fluorescence intensity of non-droplet background to ensure counting all droplets, set the threshold for 560/640 channels above the fluorescence intensity of non-fluorescent droplets to ensure counting only positive droplets.

Note: Adjust the threshold range to ensure correct counting of droplets.

b. For the droplet size, convert the droplet diameter (~ 50 μm) into image pixels, define the min/max value as 10/60 μm of the mean droplet diameter.

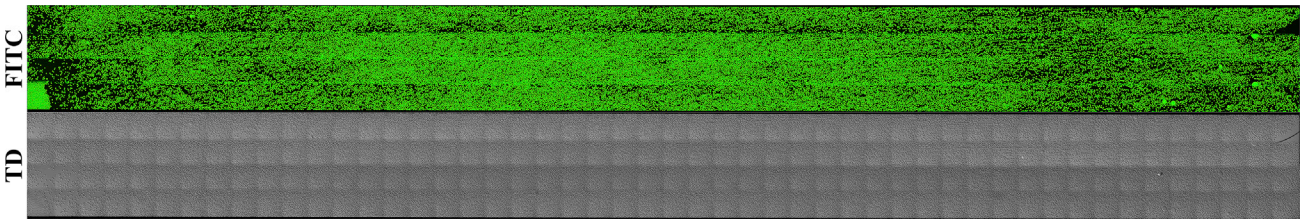

Figure 6. Whole-area images of droplet counting microwell chip
Top: FITC channel, bottom: bright-field channel.

Note: Set the min diameter to be 10 μm to ensure counting droplets around the edge of the photographs.

c. Four basic operations can be performed on the binary layer before droplet counting. Use 'Clean' to remove small objects from the binary image, 'Smooth' to smooth the binary image contours, 'Fill Holes' to fill holes within binary objects, 'Separate' to separate objects.

Note: These operations help improve the image analysis process of droplets. Perform corresponding operations if needed.

31. The number of counted droplets can be obtained from View > Analysis Controls > Automated Measurement Results. Save and export the data for future analysis (Figure 8).
32. Calculate the copy number of targets following the Poisson distribution equation: $m = n \times \ln[1/(1-p)]$, where p is the percentage of positive droplets, and n is the total number of droplets (Heyries et al., 2011; Hindson et al., 2011).

Multiplexed dLAMP evaluation (versus qPCR)

Timing: 24 h

We recommend a parallel comparison of the developed multiplexed dLAMP versus the gold standard qPCR. Plasma samples are used here to evaluate the HCV/HIV dLAMP assay.

33. Follow the manufacturer's protocol of QIAamp MinElute Virus Spin Kit to extract HCV and HIV RNA.

Pause point: Extracted/purified RNA samples can be stored at −80°C for long-term storage.

34. Reverse transcript HCV and HIV RNA into cDNAs according to the instruction of the QuantiTect Reverse Transcription Kit.

Pause point: cDNA standards can be stored at −20°C for up to 1 week.

35. Dissolve cDNA samples in 2 mL ddH_2O and divide them into two equal-volume portions. One portion will be subjected to qPCR analysis, and the other will be subjected to previously described multiplexed dLAMP assay.
36. Prepare the multiplexed qPCR reaction master mix in a microcentrifuge tube.

qPCR reaction master mix

Reagent	Final concentration	Amount
SybrGreen qPCR Master Mix (2×)	1×	10 μL
Forward primer (10 μM)	0.2 μM	0.4 μL
Reverse primer (10 μM)	0.2 μM	0.4 μL

(*Continued on next page*)

Continued		
Reagent	Final concentration	Amount
Synthetic template or cDNA	N/A	2 μL
ddH_2O	N/A	7.2 μL
Total	N/A	**20 μL**
Store at 4°C for up to 1 week.		

37. Perform the PCR in an ABI StepOnePlus qPCR thermal cycler by using the following program. And the cDNA concentrations are determined from the standard curves plotted using the synthetic templates.

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	3 min	1
Denaturation	95°C	5 s	45 cycles
Annealing/ Extension	60°C	30 s	
Hold	4°C	Forever	

38. Use the other portion for multiplexed dLAMP analysis.
39. Compare results obtained from qPCR and dLAMP to evaluate the performance of the developed dLAMP assay.

Alternatives: For RT-PCR, qPCR, and LAMP reaction, the QuantStudio 6 Flex Real-Time PCR System (ThermoFisher, Cat #4485701) and Mastercycler nexus X2 (Eppendorf, Cat #6336000023) are suggested alternatives.

EXPECTED OUTCOMES

The described protocols for multiplexed dLAMP technology provide a specific, sensitive, and robust method for the simultaneous quantification of multiple nucleic acid targets. This technology depends on the use of fluorescence activatable scorpion-shaped primers, droplet generating and counting microfluidic chips, and microscopic fluorescent droplet analysis.

Multiplexed LAMP assay

The gel electrophoresis analysis of positive LAMP products produces ladder-like bands with large molecular weights, and no amplified products for negative LAMP reaction. Positive LAMP reaction is expected to generate a typical sigmoidal real-time fluorescence curve, and no obvious fluorescence for no-template controls. The real-time fluorescence curves should be positively correlated with the concentrations of DNA targets in the range from 1.0×10^{-17} to 1.0×10^{-10} M. The limits of detection are expected to be several attomolar per reaction for both DNA targets.

Multiplexed dLAMP assay

Approximately 61,000 droplets will be collected for 2 μL sample volume. All droplets are expected to fluoresce in the FITC channel. In TAMRA and Cy5 fluorescence channels, only the positive droplets display obvious fluorescence, respectively. The fluorescence intensities of positive droplets are expected to be brightly fluoresced compared to negative droplets. The number of positive droplets should be less than 5% of the total droplets. The calculated copy numbers for DNA targets are expected to be consistent with the actual DNA copy numbers in the range from 12 to 2,400 copies. The expected limits of detection are several copies per microliter for both DNA targets. Results obtained from dLAMP and qPCR should be in good consistency (coefficient of determination, $R^2 > 0.95$).

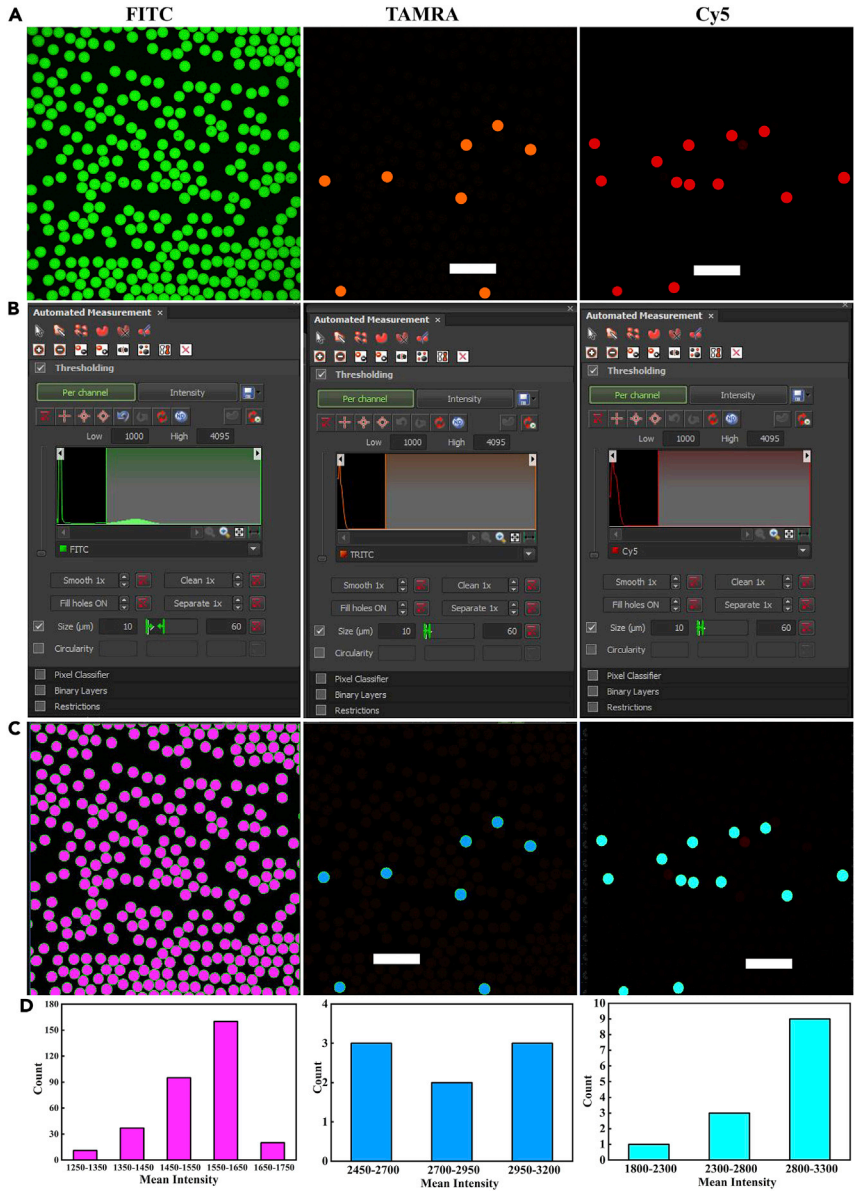

Figure 7. Droplet counting

(A) Fluorescence images of droplets. Scale bar: 200 μm.

(B) Automated measurement using NIS-Elements software.

(C) Selected and segmented droplets after automated measurement. Scale bar: 200 μm.

(D) Analysis of droplet count results.

LIMITATIONS

First, LAMP requires at least four exquisitely designed primers for each target, and more primers are needed when detecting multiple targets simultaneously, which significantly increases the difficulty in primer design and the possibility of nonspecific hybridization, leading to reduced amplification efficiency and false-positive signals. Thus, from a realistic point of view, we think this dLAMP protocol is most suitable for dual-target detection and hardly applicable for more than three targets. Secondly, this dLAMP protocol integrates a heating unit (necessary for all LAMP-based methods), a microinjection unit, and a microscopic fluorescence observation unit to fulfill analysis, not to

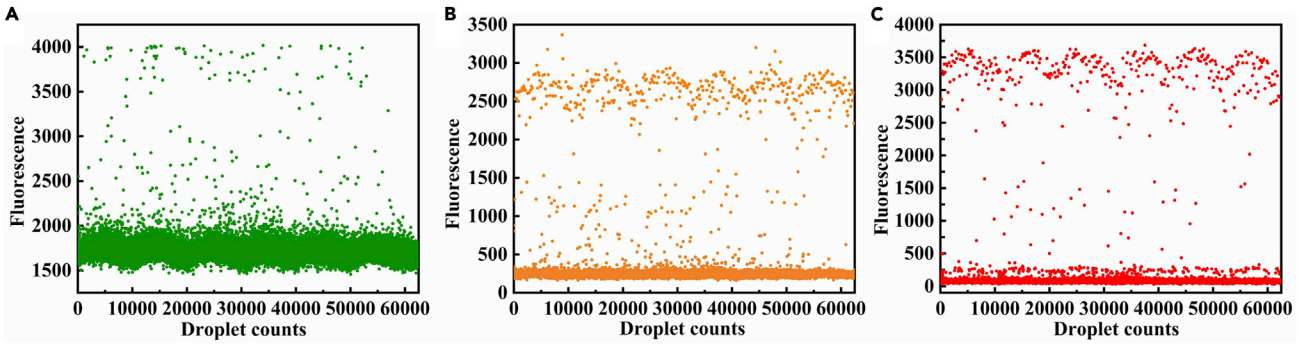

Figure 8. Scatter plot of fluorescence intensities of droplets

(A) FITC channel.

(B) TAMRA channel.

(C) Cy5 channel.

mention the preparation of complexed reagent-containing oil and aqueous phases, limiting its current application to laboratory use only.

TROUBLESHOOTING

Problem 1

The channel width of fabricated chips is inconsistent with the designed mask (step 2).

Potential solution

HF etching process is isotropic, and the channel width will continue to expand when etching downward. Therefore, pay attention when etching high-density channels, carefully control the etching conditions.

Problem 2

Hydrophobic coating layer is easy to degrade (step 3).

Potential solution

The glass channel surface is naturally hydrophilic. To form aqueous droplets in an oil phase, hydrophobic coating of the glass channel is required. The hydrophobic coating is resistant to organic solvents. But it can be removed using acidic or basic solutions, for example, a 0.1 M Sodium Hydroxide for 24 h. Therefore, do not use acidic and alkaline reagents when using the chip.

Problem 3

A positive LAMP fluorescence curve appeared in the non-template control or negative samples (step 7).

Potential solution

Potential contamination may occur during the LAMP reaction. Re-do the experiment with freshly prepared solutions to exclude reagent misuse or cross-contamination. If the false-positive result continues to exist, use 1–2% hypochlorite solution to thoroughly clean the equipment and laboratory areas. Moreover, to avoid false-positive pollution, prepare the mixture in one laboratory and add the positive target in another laboratory. And electrophoresis experiments should be done in a third laboratory.

Problem 4

The positive LAMP fluorescence curve is not detectable for the positive control (step 9).

Potential solution

The reagents or preparation of the LAMP mixture may encounter a problem. Examine reagents and enzymes, freshly prepare the mixture. In addition, check if the instrumental settings for the LAMP

reaction monitoring are correct. Analyze the LAMP reaction product with gel electrophoresis, and if no amplified product is observed, there may be a problem with the LAMP probe design.

Problem 5

The generated droplets are unstable, inhomogeneous in size, or poorly dispersed (step 19).

Potential solution

Troubleshooting priority: flow stability > flow rates of each phase > surfactant ratio > hydrophobic coating on the channel walls. In most cases, it is the problem of flow stability or flow rates. Thus, it is recommended to first stabilize the microfluidic platform. And then observe the droplet generation by adjusting the flow rates of each phase. In addition, check if there is dust or debris next to the nozzle, clean the channel and the intersection or change the microfluidic chip.

Problem 6

A multilayer emulsion is formed (step 20).

Potential solution

The hydrophobic coating of the droplet counting microwell chip is partially degraded. Use a new droplet counting microwell chip.

Problem 7

The droplets are closely packed in the counting microwell chip (step 20).

Potential solution

Reagent leakage may occur at the connection between the injectors and the injection port, or between the connection of two chips. Fix the leakage by fastening the connections.

Problem 8

Droplets are not fluorescent (step 25).

Potential solution

It may set the wrong excitation/emission parameters, adjust accordingly. Or the focal plane of the droplet is not focused, adjust the Z axis accordingly. If it is not the improper microscopic setups, there might be a problem with reagent addition. Check the reagents of the corresponding fluorescence channel, re-do the experiment.

Problem 9

Droplets are fluorescent in the non-template or negative control. Or the counted number of positive droplets is more than the estimated positive droplets (step 27).

Potential solution

It may set the wrong threshold for droplet counting, adjust the threshold parameters for accurate counting. If the problem persists, there may exist positive contamination in the experimental environment. And the solution is the same as problem 3. In addition, change a new droplet counting microwell chip if necessary.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jianjun He (jianjunh@hnu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate data sets or code for any algorithms.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101335.

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AUTHOR CONTRIBUTIONS

Y.L.T. and T.W. developed the protocol reported in this manuscript. Y.L.T., J.H.J., and J.J.H. designed this study and wrote the manuscript. All authors have reviewed the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Ball, C.S., Light, Y.K., Koh, C.-Y., Wheeler, S.S., Coffey, L.L., and Meagher, R.J. (2016). Quenching of unincorporated amplification signal reporters in reverse-transcription loop-mediated isothermal amplification enabling bright, single-step, closed-tube, and multiplexed detection of RNA viruses. Anal. Chem. *88*, 3562–3568.

Baret, J.-C. (2012). Surfactants in droplet-based microfluidics. Lab Chip *12*, 422–433.

Becherer, L., Bakheit, M., Frischmann, S., Stinco, S., Borst, N., Zengerle, R., and von Stetten, F. (2018). Simplified real-time Multiplex detection of loop-mediated isothermal amplification using novel mediator displacement probes with universal reporters. Anal. Chem. *90*, 4741–4748.

Chen, J., Xu, X., Huang, Z., Luo, Y., Tang, L., and Jiang, J.-H. (2018). BEAMing LAMP: single-molecule capture and on-bead isothermal amplification for digital detection of hepatitis C virus in plasma. Chem. Commun. *54*, 291–294.

Golozar, M., Chu, W.K., Casto, L.D., McCauley, J., Butterworth, A.L., and Mathies, R.A. (2020). Fabrication of high-quality glass microfluidic devices for bioanalytical and space flight applications. MethodsX *7*, 101043.

Heyries, K.A., Tropini, C., VanInsberghe, M., Doolin, C., Petriv, O.I., Singhal, A., Leung, K., Hughesman, C.B., and Hansen, C.L. (2011). Megapixel digital PCR. Nat. Methods *8*, 649–651.

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

Hu, F., Li, J., Zhang, Z., Li, M., Zhao, S., Li, Z., and Peng, N. (2020). Smartphone-based droplet digital LAMP device with rapid nucleic acid isolation for highly sensitive point-of-care detection. Anal. Chem. *92*, 2258–2265.

Hu, Y., Xu, P., Luo, J., He, H., and Du, W. (2017). Absolute quantification of H5-subtype Avian influenza viruses using droplet digital loop-mediated isothermal amplification. Anal. Chem. *89*, 745–750.

Jia, Z.-J., Fang, Q., and Fang, Z.-L. (2004). Bonding of glass microfluidic chips at room temperatures. Anal. Chem. *76*, 5597–5602.

Jiang, Y.S., Bhadra, S., Li, B., Wu, Y.R., Milligan, J.N., and Ellington, A.D. (2015). Robust strand exchange reactions for the sequence-specific, real-time detection of nucleic acid amplicons. Anal. Chem. *87*, 3314–3320.

Kreutz, J.E., Wang, J., Sheen, A.M., Thompson, A.M., Staheli, J.P., Dyen, M.R., Feng, Q., and Chiu, D.T. (2019). Self-digitization chip for quantitative detection of human papillomavirus gene using digital LAMP. Lab Chip *19*, 1035–1040.

Nagamine, K., Hase, T., and Notomi, T. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol. Cell Probes *16*, 223–229.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. (2000). Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. *28*, e63.

Schoepp, N.G., Schlappi, T.S., Curtis, M.S., Butkovich, S.S., Miller, S., Humphries, R.M., and Ismagilov, R.F. (2017). Rapid pathogen-specific phenotypic antibiotic susceptibility testing using digital LAMP quantification in clinical samples. Sci. Transl. Med. *9*, eaal3693.

Tan, Y.-L., Chen, H., Wu, Z.-K., He, J., and Jiang, J.-H. (2021a). Digital loop-mediated isothermal amplification-based absolute methylation quantification revealed hypermethylated DAPK1 in cervical cancer patients. Anal. Chem. *93*, 8077–8083.

Tan, Y.-L., Huang, A.Q., Tang, L.-J., and Jiang, J.-H. (2021b). Multiplexed droplet loop-mediated isothermal amplification with scorpion-shaped probes and fluorescence microscopic counting for digital quantification of virus RNAs. Chem. Sci. *12*, 8445–8451.

Tomita, N., Mori, Y., Kanda, H., and Notomi, T. (2008). Loop-mediated isothermal amplification (LAMP) of gene sequences and simple visual detection of products. Nat. Protoc. *3*, 877–882.

Varona, M., and Anderson, J.L. (2019). Visual detection of single-nucleotide polymorphisms using molecular beacon loop-mediated isothermal amplification with centrifuge-free DNA extraction. Anal. Chem. *91*, 6991–6995.

Xia, Y., Yan, S., Zhang, X., Ma, P., Du, W., Feng, X., and Liu, B.-F. (2017). Monte Carlo modeling-based digital loop-mediated isothermal amplification on a spiral chip for absolute quantification of nucleic acids. Anal. Chem. *89*, 3716–3723.

Yu, M., Chen, X., Qu, H., Ma, L., Xu, L., Lv, W., Wang, H., Ismagilov, R.F., Li, M., and Shen, F. (2019). Multistep SlipChip for the generation of serial dilution nanoliter arrays and hepatitis B viral load quantification by digital loop mediated isothermal amplification. Anal. Chem. *91*, 8751–8755.

Yu, Z., Lyu, W., Yu, M., Wang, Q., Qu, H., Ismagilov, R.F., Han, X., Lai, D., and Shen, F. (2020). Self-partitioning SlipChip for slip-induced droplet formation and human papillomavirus viral load quantification with digital LAMP. Biosens. Bioelectron. *155*, 112107.

Yuan, H., Chao, Y., Li, S., Tang, M.Y.H., Huang, Y., Che, Y., Wong, A.S.T., Zhang, T., and Shum, H.C. (2018). Picoinjection-enabled multitarget loop-mediated isothermal amplification for detection of foodborne pathogens. Anal. Chem. *90*, 13173–13177.