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A sample-to-answer COVID-19 diagnostic device based on immiscible filtration and CRISPR-Cas12a-assisted detection

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ABSTRACT

In response to the ongoing coronavirus disease 2019 (COVID-19) pandemic and disparities of vaccination coverage in low-and middle-income countries, it is vital to adopt a widespread testing and screening programme, combined with contact tracing, to monitor and effectively control the infection dispersion in areas where medical resources are limited. This work presents a lab-on-a-chip device, namely ‘IFAST-LAMP-CRISPR’, as an affordable, rapid and high-precision molecular diagnostic means for detection of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). The herein proposed ‘sample-to-answer’ platform integrates RNA extraction, amplification and molecular detection with lateral flow readout in one device. The microscale dimensions of the device containing immiscible liquids, coupled with the use of silica paramagnetic beads and guanidine hydrochloride, streamline sample preparation (including RNA extraction, concentration and purification) in 15 min with minimal hands-on steps. The pre-amplification in combination with CRISPR-Cas12a detection assays targeting the nucleoprotein (N) gene achieved visual identification of ≥ 470 copies mL −1 genomic SARS-CoV-2 samples in 45 min. On-chip assays showed the ability to isolate and detect SARS-CoV-2 RNA from 100 genome copies mL −1 of replication-deficient viral particles in 1 h. This simple, affordable and integrated platform demonstrated a visual, faster, and yet specificity- and sensitivity-comparable alternative to the costly gold-standard reverse transcription-polymerase chain reaction (RT-PCR) assay, requiring only a simple heating source. Initial testing illustrates the platform viability both on nasopharyngeal swab and saliva samples collected using the easily accessible Swan-brand cigarette filter, providing a complete workflow for COVID-19 diagnostics in low-resource settings.

1. Introduction

Since the first case of COVID-19 caused by SARS-CoV-2 was reported in Wuhan, China in December 2019, more than 600 million cases and 6.5 million deaths have been reported as of September 1, 2022 [1]. Many cases of severe illness and death associated with COVID-19 infections have been prevented in countries where there has been effective implementation of vaccine programmes, with ca. 67.7% of the world population fully vaccinated (data as of September 2021, [2]). Nevertheless, the inequitable distribution of vaccines between high and low-income countries [3], and the emergence of the highly transmissible omicron variant [4] result in 7.9 billion people living at risk of COVID-19 and thousands of deaths every week [5]. Therefore, increased testing and screening with contact tracing are paramount for infection control, especially in areas where resources are limited.

The gold standard for COVID-19 diagnostics detects the presence of viral RNA from respiratory specimens using real-time RT-qPCR [6–8]. The analytical limits of detection of RT-qPCR are usually ca. 10 3 viral

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RNA copies mL$^{-1}$ with 24-48 h turnaround time [7,9]. Despite the high sensitivity and specificity for disease diagnosis, the method relies on a highly skilled operator using expensive instrumentation, reducing its applicability for widespread application [7,10,11].

Several point-of-care (POC) RNA detection technologies, requiring no special instruments, employ simultaneous reverse transcription and isothermal amplification steps such as loop-mediated isothermal amplification (LAMP, [12,13]) or recombinase polymerase amplification (RPA, [14]). Although displaying high sensitivity, RT-LAMP and RT-RPA can suffer from nonspecific amplification under isothermal conditions, leading to false-positive results [15,16]. Other automated commercially available systems such as Cepheid GeneXpert, Roche Cobas and Abbot ID Now offer the ease-of-use aspect with minimal hands-on steps with $\leq 1$ h sample-to-result; however, expensive specialized instrumentation is still required for each system [17].

As an alternative to nucleic acid amplification tests, antigen lateral flow assays detect the presence of the virus through specific nucleocapsid proteins of SARS-CoV-2. They require no RNA purification step, and can operate at ambient temperatures with $\leq 30$ min turnaround time, making them appealing within POC settings. However, the lack of exponential amplification can hinder the detection limit with a sensitivity of the assays typically being three orders of magnitude lower than the gold standard RT-qPCR [18].

Clustered regularly interspaced short palindromic repeats (CRISPR)-based diagnostic methods utilize the collateral cleavage activity of bystander nucleic acid probes of RNA-guided CRISPR-associated 12/13 (Cas12/13) nucleases [16,19-22]. In combination with RT-LAMP or RT-RPA isothermal amplification methods, CRISPR-Cas-assisted SARS-CoV-2 detection assays are considered as transformative methods for POC COVID-19 diagnostics [16,22-26]. The highly sensitive and specific nature of CRISPR diagnostic methods is attributed to the sequence specificity required for both nucleic acid amplification step and the CRISPR-Cas detection step [16]. Readout of Cas-mediated nucleic acid probe cleavage can either be by fluorescence detection or via lateral flow strip, both of which can be applied for POC purposes [22,27]. The original DETECTR-based [22], and SHERLOCK-based [16] detections involved multiple manual steps and operated with already extracted RNA, thereby limiting their applicability for POC use [28]. Although the more recent STOPCovid.v2 assay included a benchtop magnetic-based RNA extraction prior to the one-step CRISPR-Cas12b-assisted RT-LAMP [29], the assay still requires multi-step manual operation.

Numerous efforts have been made in developing fully integrated, affordable, sensitive, specific, rapid and robust platforms for POC COVID-19 diagnostics exploiting microfluidics or ‘lab-on-a-chip’ technologies [17,30]. Such platforms permit viral lysis, RNA extraction, nucleic acid amplification and detection on a single device, allowing more applicable diagnostics within resource-limited settings [28,31]. Immiscible filtration assisted by surface tension (IFAST) is a microfluidic technique which exploits the surface tension properties of immiscible liquids (e.g., aqueous and oil) and microscale dimensions to facilitate side-by-side compartmentation of immiscible liquids, enabling multiple and sequential steps to be performed in one device [32-34]. The use of suitable functionalized magnetic particles allows rapid isolation and purification of a magnetically responsive analyte from complex matrices.
Here, IFAST, LAMP and the recent developments in CRISPR-Cas12-based sensing applied to SARS-CoV-2 detection are combined to develop a cost-effective, sensitive, target-specific and fully integrated device for COVID-19 diagnostics, namely ‘IFAST-LAMP-CRISPR’, Fig. 1A. The device was designed for streamlined sample preparation to provide rapid isolation and concentration of RNA directly from nasopharyngeal swabs or saliva specimens followed by pivotal RT-LAMP and CRISPR-Cas-assisted detection with lateral flow readout. The versatility of the developed platform can be implemented for CRISPR-Cas-based detections of other pathogens, showing great promise for POC diagnostics particularly in decentralized and resource-limited settings in low- and middle-income countries.

2. Materials and methods

2.1. Ethical approval

This study was approved by the Mount Kenya University Independent Ethical Review Committee (MKU/IERC/1811) and performed in accordance with relevant guidelines and regulations. Nasopharyngeal swab and saliva samples were collected from participants with their written informed consent after the nature and possible consequences of the study had been fully explained to them.

2.2. Materials and reagents

Genomic SARS-CoV-2 RNA (2019-nCoV/USA-WA1/2020, ATCC VR-1986D), HCoV-Oc43 (ATCC VR-1558D) and H1N1 (ATCC VR-1736D) RNAs were purchased from LGC standards, UK. SARS-CoV-2 verification panel (AccuPlex SARS-CoV-2, 0505-0168) was supplied by Technotaph, UK. LAMP primers and lateral flow reporter were purchased from Integrated DNA Technologies (IDT). SYBR Safe, nuclease-free water, RNase decontamination solution and PCR adhesive film were supplied by Thermofisher Scientific, UK. Guanidine hydrochloride (GuHCl) and MagneSil paramagnetic beads were purchased from Promega. Lateral flow strips (Milenia HybriDetect 1) were purchased from TwistDx, UK.

2.3. Device design, fabrication and preparation

The microfluidic device (Fig. 1B) was made of polymethyl methacrylate and fabricated via CNC machine milling (Datron M7, Milton Keynes, UK). The device features a large sample chamber (1) (w = 15 mm, l = 26 mm); wash chambers (2), (4), (6) (w = 3 mm, l = 3 mm); wash chambers (3), (5) (w = 3 mm, l = 8.5 mm); RT-LAMP chamber (7) (w = 3 mm, l = 3 mm); chamber (8) (w = 3 mm, l = 15 mm); and a CRISPR-Cas detection chamber (9) (w = 6 mm, l = 9 mm). All chambers were 3.8 mm deep, and were interconnected via gates (gate from sample chamber to chamber 2: w = 3.5 mm to 3 mm with 0.5 mm restriction; gates between chambers 2 to 9: w = 3 mm to 3 mm with 0.5 mm restriction; all gates: l = 3 mm, d = 0.2 mm), Fig. 1B and C. The sample chamber contained a syringe port (Ø = 4 mm) for a Luer fitting for sample introduction.

Employing the same material with similar chamber and gate dimensions to the single-assay device, some modifications were made in order to facilitate multiplexing assays (Fig. S1, Supplementary Materials).

For development of prototype devices in the UK, devices were cleaned with RNase decontamination solution, followed by rinsing with nuclease-free water, and were left to dry at ambient temperature prior to use. The bottom of the device was sealed with PCR adhesive film. For clinical validations carried out in Kenya, devices were cleaned with ethanol and left to dry, and sealed with PCR adhesive film prior to use.

2.4. Tube-based RT-LAMP, followed by CRISPR-Cas12-assisted detection (DETECTR assays)

Tube-based DETECTR assays were performed using RT-LAMP for pre-amplification of genomic SARS-CoV-2 RNA using primers targeting N gene and LbCas12a for the trans-cleave assay following the protocol described by Broughton et al. [22] with some modifications. Details of primers and guide RNAs are summarised in Table S1, Supplementary Materials. RT-LAMP was conducted at 64°C for 30 min using 2 μL RNA templates serially diluted (10x) from 4,700 copies μL⁻¹. RT-LAMP products were identified via agarose gel electrophoresis (1 %w/v agarose, 1xTAE buffer, 80 V, 45 min) and the results analyzed using a molecular imager (Chemidoc XRS+, BioRAD). LbCas12a-gRNA complexes were generated prior to carrying out LbCas12a trans-cleave assay, by pre-incubating LbCas12a (50 nM) with gRNA (62.5 nM) in 10xNEBuffer 2.1 at 37°C for 30 min, followed by an addition of the lateral flow cleavage ssDNA reporter (56-FAM/TTATTATT/3Bio/) to the reaction at a final concentration of 500 nM. CRISPR-Cas12-assisted detection was performed by mixing 2 μL of RT-LAMP amplicons with 22 μL of RNP complex containing lateral flow cleavage reporter and 76 μL of 1xNEBuffer 2.1, and incubating at 37°C for 10 min. Subsequently, a lateral flow strip was added to the reaction tube and the result was visualized/photographed after ca. 2 min using a smartphone camera (Samsung Galaxy A3). A negative sample was identified by the presence of a single band close to the sample pad (control line – C), whereas the presence of a single band close to the top of the test strip (test line – T), either with or without a control line, indicated a positive result (Fig. 1D). The gRNA specificity was evaluated against HCoV-Oc43 and H1N1 RNAs using their respective primers [34].

2.5. On-chip RNA extraction and RT-LAMP (Steps 1&2, Fig. 1A)

A sample with/without SARS-CoV-2 RNA was diluted in 5 M GuHCl containing 0.005% Tween 20 and added to the sample chamber (1) together with Magnesil paramagnetic beads (MBs, 1 μL). The mixture was gently agitated by hand or using a rotator set at 40 rpm (Stuart SB3, UK) for 5 min. Next, empty chambers were filled with mineral oil (chambers (2), (4), (6), (8)); RT-LAMP reagent overlaid with mineral oil (chamber (7)) and 0.005% Tween 20 (chambers (3), (5), (9)). The RNA-bound MBs were gathered, via the use of an external NdFeB magnet assembly (a 20 mm × 10 mm × 5 mm bar magnet and a 4 mm diameter × 5 mm height disc magnet; magnetic strength = 0.42 Tesla, Magnet Sales, UK) in the sample chamber, washed through wash liquids in chambers (2)-(6) and mixed with RT-LAMP reagent in chamber (7). The chip was subsequently placed in an incubator for RT-LAMP reaction at 64°C for 30 min. The RT-LAMP amplicons (2 μL) were added into a tube containing CRISPR-Cas12 reagents (22 μL RNP complex containing lateral flow cleavage reporter and 76 μL 1xNEBuffer 2.1) and the reaction incubated at 37°C for 10 min, prior to result visualization via lateral flow strip.

2.6. On-chip CRISPR-Cas12 detection assays (Step 3, Fig. 1A)

The capability of the device for CRISPR-Cas12 detection assays was tested on tube-based RNA-extraction and RT-LAMP amplicons. Magnesil paramagnetic beads (1 μL) were added into a sample of genomic SARS-CoV-2 RNA diluted in 5 M GuHCl (940 copies μL⁻¹) and mixed for 5 min by tube inversion. The RNA-extracted MB were washed with nuclease-free water (100 μL). The washed RNA-extracted MB were mixed with RT-LAMP reagent to undergo reaction at 64°C for 30 min. The amplicons (2 μL) were transferred from the reaction tube into chamber (9) of the chip device which was pre-filled with CRISPR-Cas12 assay mix and sealed with PCR adhesive film on the top to prevent contamination. Other chambers were also alternately filled with nuclease-free water (chambers (1), (3), (5), (7)), and mineral oil (chambers (2), (4), (6), (8)). On-chip CRISPR-Cas12 assays were performed in an incubator at 37°C
for 10 min.

2.7. Combined on-chip RNA extraction, RT-LAMP and CRISPR-Cas12-assisted detection (Steps 1-3, Fig. 1A)

On-chip RNA extraction and RT-LAMP were similarly conducted as described above. Extraction was performed for 5 min for samples containing free RNAs, and 15 min for SARS-CoV-2 verification panel samples. After performing RT-LAMP at 64°C for 30–45 min, CRISPR-Cas12 reagent mix was added to chamber (9) and MBs from chamber (7) were moved into chamber (9) and the chamber was sealed with PCR adhesive film prior to reaction at 37°C for 10 min and 2 min for lateral flow readout.

2.8. Clinical sampling

Clinical samples from symptomatic COVID-19 infected patients (25-40 years old) confirmed by RT-qPCR were retrieved from a national testing centre at Kenya Medical Research Institute. The sampling was authorized by the Ethical Review Committee of Mount Kenya University (MKU/IERC/1811) and carried out in accordance with the approved guidelines. For saliva collection, patients were asked to place a Swan cigarette filter under their tongue. Both the nasopharyngeal swabs and saliva samples in the filters were placed in Viral Transport Media (VTM) and stored at -80°C at Mount Kenya University for use.

2.9. IFAST-LAMP-CRISPR device for COVID-19 detection in clinical samples

Collected saliva samples were first screened for SARS-CoV-2 status using tube-based RT-LAMP followed by gel electrophoresis. RNA was first extracted using the Ribo-Virus extraction kit following manufacturer’s instruction. One micro litre of the extracted RNA was then subjected to RT-LAMP using WarmStart® LAMP Kit (New England Biolabs) and the amplification products analysed using a 1%w/v agarose gel electrophoresis. The samples that turned positive were used in the subsequent experiments for on-chip RNA extraction, RT-LAMP and CRISPR-Cas12-assisted detection.

For on-chip integrated RNA extraction, RT-LAMP and CRISPR-Cas12a assisted detection, a saliva sample (50 μL) was mixed with 5 M GuHCl (949 μL), 2.5%w/v Tween 20 (2.5 μL) and Magnesil paramagnetic beads suspension (1 μL). The mixture was gently agitated by hand for 15 min. Next, chambers 2 and 4 were filled with 20 μL mineral oil while chamber 8 was filled with 100 μL mineral oil. Chambers 3 and 5 were filled with 50 μL 5 M GuHCl containing 0.005%w/v Tween 20, and RT-LAMP reagents were added into chamber 8. After 15 minutes of incubation, the RNA-bound MBs were gathered, via the use of an external NdFeB magnet assembly in the sample chamber and washed through wash liquids in chambers (2-6) and mixed with RT-LAMP reagent in chamber 7. The chip was subsequently placed on a heat block for RT-LAMP reaction at 64°C for 30 min. After amplification, the MBs were moved into chamber 9 which was prefilled with CRISPR-Cas12 assay mix. The mixture was incubated at 37°C for 15 min prior to result visualization via a lateral flow strip.

2.10. Multiplex detection using multiplexing IFAST-LAMP-CRISPR device

The single-assay IFAST-LAMP-CRISPR device was modified to facilitate multiple detections from one sample (Fig. S1, Supplementary Materials). The sample chamber was fitted with three gates leading off to individual assay specific lines. On-chip integrated steps were carried out for detection of N and E genes using the primers reported by Broughton et al. [22]. For proof-of-concept experiments, the wash chambers of all three assay lines were alternately filled with 0.005% Tween 20 and mineral oil, whilst the sample chamber was filled with 5 M GuHCl containing 0.005% Tween 20 and 3 μL Magnesil paramagnetic beads. The device was agitated for 1 min prior to magnetic beads gathering into three portions aligning with the three assay specific lines, using three separate disc NdFeB magnets (ø = 4 mm, h = 2 mm) attached to a bar NdFeB magnet (w = 10 mm, l = 20 mm, h = 5 mm). The bead population on the upper position was pulled through wash liquids and stored in mineral oil inside chamber 6, and was subsequently used as negative control. Afterwards, a specific volume of SARS-CoV-2 RNA or SARS-CoV-2 verification panel was added to the sample chamber to afford the desire concentration, and the device was manually agitated for 10 min. Portioning of the remaining magnetic beads into two groups (middle and lower groups) were next conducted, using two NdFeB disc magnets, followed by washing through immiscible liquids and stored in the corresponding chamber 6 of the middle and lower lines. Next, RT-LAMP reagents with N or E gene primers were added into chamber 7 of each line (upper and middle lines = N gene, lower line = E gene), and overlaid with 10 μL mineral oil. The beads from chamber 6 of each line were simultaneously pulled into chamber 7. The chip was placed in an incubator set at 64°C for 30 min. Subsequently, CRISPR-Cas12a-assisted detection were carried out as previously described using respective gRNAs.

2.11. Lyophilized reagents

To the RT-LAMP reaction mix excluding isothermal amplification buffer, 38.8%w/v D-trehalose (Sigma-Aldrich) was added to afford a final concentration of 10%w/v. This master mix was aliquoted (11.6 μL) into DNase/RNase-free tubes or chamber 7 of the IFAST-LAMP-CRISPR device, and stored at -80°C for 1 h prior to freeze-drying at -45°C, 11.6 psi for 24 h. The lyophilized reagent was kept at -20°C. For RT-LAMP, the lyophilized reagent was re-constituted in 12.5%w/v isothermal amplification buffer (20 μL, New England Biolabs) and the reaction was conducted as previously described.

For lyophilized CRISPR-Cas12a reagents, nuclelease-free water was replaced with 12.7%w/v D-trehalose (final concentration = 10%) in the master mix, which was subsequently aliquoted into DNase/RNase-free tubes (20 μL) and kept at -80°C for 1 h prior to freeze-drying at -45°C, 11.6 psi for 24 h. The lyophilized reagents were kept at -20°C and reconstituted in nuclelease-free water (20 μL) before use.

On-chip integrated steps were next conducted in a single assay device containing lyophilitized RT-LAMP reagents within chamber 7 in a similar manner as in the assay with freshly prepared reagents. After amplification, the magnetic beads were pulled into chamber 9 prefilled with CRISPR-Cas12 assay mix prepared from lyophilized reagents and the detection process continued as previously explained.

3. Results and discussion

3.1. Development of IFAST-LAMP-CRISPR microfluidic device for SARS-CoV-2 detection

The IFAST-LAMP-CRISPR device (Fig. 1B and C) has been designed to combine streamlined IFAST sample preparation for rapid and effective RNA extraction from viral particles, with sensitive and highly specific CRISPR-Cas-associated detection. The design was adapted from our previously reported IFAST RT-LAMP device [34]; however, in this instance, the chambers were horizontally aligned for easier magnetic manipulation, multiplexing and automation (Fig. 1C); as opposed to the meandering chamber layout in the previous design which was chosen in order to minimise carry-over and device footprint. The gates and interconnecting chambers were of similar dimensions to those of the previous design (Fig. 1B), permitting up to 60 min heating at 65°C with intact immiscible interfaces [34]. The operation steps for SARS-CoV-2 detection utilizing the IFAST-LAMP-CRISPR device are (i) loading the sample chamber with viral sample diluted in 5 M GuHCl and incubating the sample mix with silica paramagnetic beads, either by manual agitation or using a shaking/rotating platform, (ii) adding oil, washing...
liquid and RT-LAMP reagent, (iii) isolating the magnetic beads from the sample and washing through immiscible liquids using magnetic manipulation across the bottom of the device, to allow washed magnetic beads to mix with RT-LAMP reagents, (iv) performing RT-LAMP by placing the device in an incubator or over a hot plate, (v) adding CRISPR-Cas12a reagent in the last chamber and moving the magnetic beads from the RT-LAMP chamber into the CRISPR-Cas12 detection chamber, (vi) carrying out CRISPR-Cas12a detection in an incubator/on a hot plate, (vii) adding a lateral flow test strip into the CRISPR-Cas12a chamber for result visualization.

The platform simplified the cumbersome RNA extraction process normally required prior to performing CRISPR-Cas-based assays for SARS-CoV-2 detection [41]. This was achieved by combining the lysis and magnetic bead-binding steps, and eliminating the ethanol wash and elution steps typically conducted in multi-step solid phase RNA extraction. The duration of the sample extraction time was reduced to just 15 min with minimal hands-on time. The relatively large sample chamber accommodating up to 1.5 mL sample, coupled with the concentration of analyte by magnetic beads, will benefit the detection of low viral loads. Magnetic beads were chosen over oligo (dT)-functionalized magnetic beads employed in the previous IFAST LT-LAMP device [34], in order to reduce the cost per assay (Table S1, Supplementary Materials). Additionally, Manganese Silica magnetic beads can be stored at room temperature (15–30°C), making them more suitable for limited-resource settings than oligo (dT)-functionalized beads which require a cold chain storage (2–8°C). The chaotropic salt GuHCl was used in the lysis and binding step as well as acting as RNAse inhibitor to help maintain RNA integrity in clinical samples. Inexpensive GuHCl salt can be pre-stored inside the device in a solid form, or directly used as GuHCl (aq).

By passing RNA-bound MBs through two sets of immiscible wash liquids, rapid purification of the magnetic bead-isolated RNA was achieved within a few seconds, thereby protecting the RNA integrity for the subsequent amplification and detection steps.

The following amplification and detection steps were adapted from the reported SARS-CoV-2 DETECTr protocol from Broughton et al. [22] where simultaneous reverse transcription and isothermal amplification were achieved by RT-LAMP, followed by CRISPR-Cas12 detection of target DNA amplicons. As well as being affordable and widely accessible, the reported LAMP N gene primer set was also granted Emergency Use Authorization (EUA) by the U.S. Food and Drug Administration for Mammoth Biosciences [43]. Despite being active at 16–48°C, the Cas12a enzyme (Cpf1) is commercially available and more accessible than the thermo-stable AapCas12b exploited in the STOPcovid system [29]. Tube-based CRISPR-Cas assays require the transfer of minute quantities of DNA target from the amplification tube into the CRISPR-Cas detection tube [16,22]. By including an additional oil chamber after the RT-LAMP chamber on the IFAST-LAMP-CRISPR device, magnetic manipulation of amplicons-bound on MBs into the CRISPR-Cas detection chamber can easily be achieved, preventing potential contamination introduced by amplicon transfer by pipetting.

A lateral flow test strip was used for result interpretation instead of fluorescence detection to avoid utilizing a complex equipment set up. The test strips embedded biotin-ligand and anti-FITC/FAM antibodies in the control and test lines, respectively (Fig. 1D). The ssDNA lateral flow reporter was labelled on the 5'-end with FAM and on the 3'-end with biotin. If no target DNA is present (SARS-CoV-2 negative sample), ssDNA reporters will hybridize to the control line close to the sample application pad. While on the contrary, positive samples containing target DNA which when hybridized with the gRNA, will trigger collateral cleavage of the ssDNA reporters in the proximity, activating a test line closer to the end of the test strip. This unambiguous detection step takes 1-2 min, and results can be analysed by the naked eye, facilitating device deployment in resource-poor settings where readily and accurately interpreted results are necessary.

3.2. CRISPR-Cas-assisted detection of SARS-CoV-2

Validation of the CRISPR-Cas12a-based assays for SARS-CoV-2 detection was first carried out in tubes on a series of ten-fold dilutions of genomic RNA with initial concentration of 4,700 copies mL\(^{-1}\). Utilizing primers targeting N gene, positive amplifications were observed from just 5 copies of genomic RNA after 30 min (Fig. 2A, gel electrophoresis); a much higher sensitivity compared with the pH-dependent colorimetric RT-LAMP assay reported in our previous study where ≥470 genome copies were amplified at the same reaction time [34]. Visual signals observed from 10 min CRISPR-Cas12 detection in the following step confirmed the sensitivity of the assay to detect down to 5 genome copies (Fig. 2A, lateral flow strips). The specificity of the gRNA in the CRISPR-Cas12 assay only targeted SARS-CoV-2 with no cross-reactivity with the related HCoV-OC43 and influenza A H1N1 demonstrated in Fig. 2B. Although positive amplifications were detected with correct pairing between RNAs and their respective primers (Fig. 2B, gel electrophoresis), a test line only appeared on the strip from the SARS-CoV-2 sample as a result of Cas12a trans-cleavage activity following hybridization of the gRNA-targeted DNA sequence.

The CRISPR-Cas-assisted assay was further tested with RNA extracted from nasopharyngeal swab and saliva samples (Fig. 2C), demonstrating successful detection of SARS-CoV-2 RNA in RT-qPCR confirmed positive samples.

3.3. Evaluation of the IFAST-LAMP-CRISPR device for SARS-CoV-2 detection

In order to apply the IFAST-LAMP-CRISPR device for SARS-CoV-2 detection, individual steps on-chip were systematically evaluated. Utilizing silica paramagnetic beads combined with guanidinium salt, genomic SARS-CoV-2 RNA was extracted and purified through the immiscible liquids, and finally underwent RT-LAMP on chip. The amplification products were subsequently detected via tube-based CRISPR-Cas12 assay. Verification of target DNA amplicons successfully generated from magnetically-isolated RNA from initial samples containing ≥470 genome copies mL\(^{-1}\) is demonstrated in Fig. 3A.

The feasibility of performing a CRISPR-Cas12a assay for the specific detection of target DNA on-chip was next investigated. The corresponding readout signals from negative and positive samples suggested the viability of the IFAST-LAMP-CRISPR device as a reactor for the trans-cleavage activity of Cas12a to detect target amplicons (Fig. 3B).

The performance of the IFAST-LAMP-CRISPR device was next examined on the combined workflow for extraction, RT-LAMP and CRISPR-Cas12a detection of samples containing genomic RNA (Fig. 3C–top). Negative sample for SARS-CoV-2 containing a mixture of HCoV-OC43 and H1N1 RNAs showed only a control line on the lateral flow test strip, whilst test lines were present from positive samples containing a mixture of HCoV-OC43, H1N1 and ≥470 copies mL\(^{-1}\) SARS-CoV-2 RNAs. These results not only validated the successful integration of consecutive steps on-chip, but they also confirmed the assay’s specificity as previously described in Fig. 2B. The LoD of our device is much lower than the average viral loads reported in clinical samples (saliva: 10^3 – 10^5 copies mL\(^{-1}\) [44]; sputum: 5 × 10^3 copies mL\(^{-1}\) [45], 7.5 × 10^5 copies mL\(^{-1}\) [46]; throat swab: 8 × 10^4 copies mL\(^{-1}\) [46]). The assay performance of the IFAST-LAMP-CRISPR device was further evaluated using replication-deficient SARS-CoV-2 viral particle samples (SeraCare AccuPlex SARS-CoV-2 Verification Panel). By mixing the viral sample with silica paramagnetic beads and 5 M GuHCl, lysis of viral particles and binding of SARS-CoV-2 RNA to magnetic beads were accomplished simultaneously (Fig. S2A, Supplementary Materials). Translating the same process onto the IFAST-LAMP-CRISPR device, coupled with rapid washing through minute quantities of immiscible liquids, demonstrated that RNA can be magnetically isolated and detected from contrived viral samples containing 100 genome copies mL\(^{-1}\) in 15 min (Fig. 3C–bottom). RT-LAMP DNA amplicons generated from the magnetically
isolated RNA, verified by gel electrophoresis, confirmed the efficient on-chip purification process in eliminating possible RT-LAMP assay inhibitor crossing over from the sample chamber. Finally, recognition of target DNA sequences that had been magnetically transported to the CRISPR-Cas12a detection chamber was performed. This was achieved by using the Cas12a gRNA initiated collateral cleavage of ssDNA repressor, leading to a test line on the lateral flow strip, similar to the tube-based assay for positive control of free SARS-CoV-2 RNA. The here described simple, and yet complete on-chip workflow has already shown a similar level of detection as the gold standard RT-qPCR. It is also similar to the 100 copies mL⁻¹ LoD reported using the Abbott Real Time SARS-CoV-2 Emergency Use Authorization test, where only 5% positive cases would be missed [47].

3.4. Easy access and cost-effective cigarette filters for saliva collection

Another aim of the present study was to replace the uncomfortable nasopharyngeal (NP) or oropharyngeal (OP) swabbing with saliva sampling using a cigarette-type filter. Although NP and OP swab specimens have been considered reliable and are most often used for COVID-19 diagnostics [5,7], acquiring an NP/OP swab can be unpleasant, and can initiate coughing leading to increasing risk of nosocomial spread of respiratory viruses [48], as well as in suboptimal specimens being collected dependent upon the experience of the collector. The inexpensive and easily accessible Swan brand cigarette filters were investigated as a potential substrate for saliva collection for COVID-19 screening in low-resource settings. The feasibility of introducing a viral sample from a Swan cigarette filter for the IFAST-LAMP-CRISPR device was examined using genomic SARS-CoV-2 RNA (Fig. S3, Supplementary Materials). No adverse effect from the filters was observed either on RT-LAMP or CRISPR-Cas12a assays performed on-chip. Additionally, Swan brand filters were tested with dilutions of a SARS-CoV-2 positive nasopharyngeal swab specimens. After immersing a filter in 1 mL of each dilution, the liquid retained within the filters was extracted and
subjected to RT-qPCR. Ct values obtained from the samples retained within the filters compared favourably to those of the initial samples without filters, indicating no RT-qPCR assay inhibition, no significant retention of viral particles in the filters, or compromised RNA integrity by the use of Swan filters (Fig. S3, Supplementary Materials). These proof-of-concept methodological approaches verify the potential use of Swan filters for saliva collection, which can be directly interfaced with the IFAST-LAMP-CRISPR device.

3.5. Testing of IFAST-LAMP-CRISPR device on clinical samples

The IFAST-LAMP-CRISPR device was first evaluated with nasopharyngeal swab samples. Prior to performing the integrated steps, the samples were subjected to on-chip RNA extraction and RT-LAMP in order to validate the viability of the on-chip sample preparation steps. Comparative results between tube-based assays using RNA extracted from a positive sample and on-chip assays from crude sample indicate successful on-chip RNA extraction (Fig. S2B, Supplementary Materials). Effective purification of the magnetic beads-captured RNA through immiscible liquids in the wash chambers was confirmed by the positive amplification (RT-LAMP chamber). This demonstrates great promise in improving the usability of CRISPR-based COVID-19 diagnostics for field applications. Sample preparation has been the bottleneck for CRISPR-assisted detection, requiring time-consuming and equipment-intensive nucleic acid isolation [41]. Although ultrasensitive and high specificity microfluidic CRISPR-based COVID-19 diagnostic devices have successfully been developed e.g., RApid Digital CRISPR Approach (RADICA) [49], digital warm-start CRISPR (WS-CRISPR) [50], digitization-enhanced CRISPR/Cas-assisted one-pot virus detection (deCOVID) [51], clinical samples were still required to be extracted and purified through lengthy off-chip processes. Our platform utilizes silica binding in the presence of guanidinium salt, enabling rapid isolation of RNA within 15 min. On-chip integrated steps with lateral flow readout showed positivity (indicative test lines) on RT-qPCR-confirmed positive nasopharyngeal samples with Ct values of 3.96 and 11.53 (Fig. 4A) within 1 h from sample-in to answer-out (15 min extraction, 30 min pre-amplification and 15 min CRISPR-Cas12a assisted detection). In comparison with the remarkably fast on-chip electric field and microfluidic isotachophoresis-CRISPR (ITP-CRISPR) assay (<40 min for nucleic acid extraction and purification, and SARS-CoV-2 detection by CRISPR) [52], our platform integrated all steps in one device, whereas the ITP-CRISPR assay required off-chip manual steps for RT-LAMP and sample lysis. The double selective screening by both RT-LAMP primers and CRISPR guide RNA of our IFAST-CRISPR assay prevents false positive results commonly associated with only RT-LAMP-based assays [53, 54]. Although less robust, compared to the commercially available automated Eua tests e.g., Abbot ID Now COVID-19 or Cepheid Xpert Xpress SARS-CoV-2 molecular test, our platform can be more economically deployed for point-of-care testing in resource-constrained settings.

Having previously demonstrated the ability to maintain RNA integrity as well as the compatibility of the Swan filter to the downstream RT-LAMP and CRISPR-Cas12a assisted assays, on-chip SARS-CoV-2 detection was next assessed with saliva samples collected in Swan filters stored in VTM. Similar to the nasopharyngeal swab samples, SARS-CoV-2 RNA was successfully extracted from saliva samples utilizing our IFAST-LAMP-CRISPR device (Fig. S2C, Supplementary Materials). The fully integrated on-chip steps revealed a marked distinction between no template control and positive saliva samples (Ct values of 13.13, and 16.38, Fig. 4B). This result suggests that our proposed platform can be applied for COVID-19 screening on different types of samples without modification.

Additionally, the inexpensive and easily available Swan filters avoid consequences from supply shortages of swabs and enable more comfortable sample collection, thus reducing disease transmission from sneezing/coughing introduced by irritation during nasopharyngeal swabbing. In order to overcome the main challenges during platform
testing with clinical samples in Kenya, e.g., reagent accessibility and compromised enzyme activities due to delay in delivery, freeze-drying of RT-LAMP and CRISPR-Cas12a reagents was attempted using 10% w/v D-trehalose as a lyoprotectant. Similar reactivities to the freshly prepared reagents were obtained from both lyophilized RT-LAMP and CRISPR-Cas12a reagents after up to two weeks (Figs. S4 & S5, Supplementary Materials). On-chip integrated steps tested with SARS-CoV-2 RNA using a device in which the RT-LAMP chamber was pre-loaded with reagents (on-chip lyophilization) also displayed positive amplification of targeted RNA (Fig. S6A, B, Supplementary Materials). The presence of the test line in the lateral flow test strip indicated the viability of the lyophilized CRISPR-Cas12a reagents in the subsequent detection step (Fig. S6C, Supplementary Materials), suggesting the feasibility of reagent pre-storage in the IFAST-LAMP-CRISPR device for ease of use for field deployment.

### 3.6. IFAST-LAMP-CRISPR device for multiplexing assays

The initial design for multiplexing the IFAST-LAMP-CRISPR device exploited the specificity of the RT-LAMP primers and CRISPR gRNAs to enable simultaneous multiplexed readout from one single sample. By paralleling arrays of single-assay lines, the device permitted differentiation of two genomic targets of SARS-CoV-2 RNA (N and E genes) from one sample in one hour (Fig. 5A). Although not investigated here, future clinical validation could employ human RNase P in the upper line as the extraction process control [6]. The application of our multiplexing IFAST-LAMP-CRISPR device is not limited only to genomic target identification, the distinction between different strains of coronavirus and diseases with similar symptoms, as well as the imperative screening for new SARS-CoV-2 variants of concern are also feasible [55].

### 4. Conclusion

We have demonstrated the manual operation of an IFAST-LAMP-CRISPR device, for use as a molecular COVID-19 diagnostic tool, by a semi-trained operator within resource-constrained laboratories in sub-Saharan Africa, e.g., Kenya. By incorporating the powerful CRISPR-Cas12 diagnostics with the rapid and robust lab-on-a-chip sample preparation and pre-amplification methodology, all essential steps for SARS-CoV-2 detection can be achieved within one single device. The device has been designed to operate with already available laboratory facilities such as incubator and hot plate for heating, eliminating the need for costly or specialized instruments. It is acknowledged that more clinical validation is required in order to quantify the performance of the...
herein proposed platform with respect to the gold-standard RT-qPCR method. However, the RT-qPCR comparative sensitivity and specificity obtained from such a device tested with replication-deficient viral particles, as well as the proof-of-concept results from clinical nasopharyngeal swab and saliva samples, illustrate its great potential use for detection of SARS-CoV-2 and other related infections. By adapting the single-assay IFAST-LAMP-CRISPR design, we have demonstrated simultaneous detections of N- and E-genes of SARS-CoV-2 RNA from one sample. The total cost of a device including detection, materials and reagents is currently around 10 USD (Table S2, Supplementary Materials), although this could be reduced if the devices are mass produced. This simple, and yet highly versatile platform can present an affordable method. However, the RT-qPCR comparative sensitivity and specificity obtained from such a device tested with replication-deficient viral par

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.talanta.2022.100166.

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