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Article

Optimization of Reverse Transcription Loop-Mediated Isothermal Amplification for In Situ Detection of SARS-CoV-2 in a Micro-Air-Filtration Device Format

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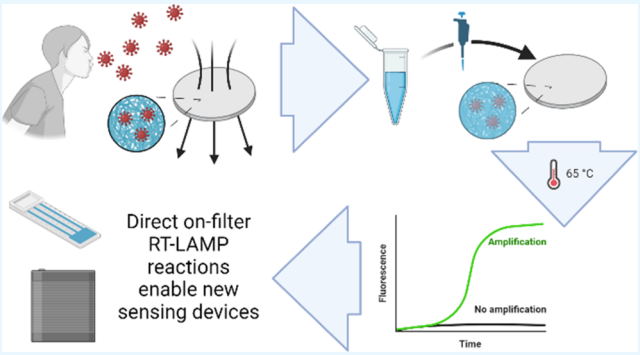
ABSTRACT: The Coronavirus disease 2019 (COVID-19) pandemic has supercharged innovation in the field of molecular diagnostics and led to the exploration of systems that permit the autonomous identification of airborne infectious agents. Airborne virus detection is an emerging approach for determining exposure risk, although current methods limit intervention timeliness. Here, we explore reverse transcription loop-mediated isothermal amplification (RT-LAMP) assays for one-pot detection of Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) (SCV2) run on membrane filters suitable for micro-air-filtration of airborne viruses. We use a design of experiments statistical framework to establish the optimal additive composition for running RT-LAMP on membrane filters. Using SCV2 liquid spike-in experiments and fluorescence detection, we show that single-pot RT-LAMP on glass fiber filters reliably detected 0.10 50% tissue culture infectious dose ($TCID_{50}$) SCV2 per reaction (3600 E-gene copies) and is an order of magnitude more sensitive than conventional RT-LAMP.



INTRODUCTION

The rapid and reliable detection of airborne pathogens is important for effective disease management and control. Polymerase chain reaction (PCR) is a gold standard molecular diagnostic technique due to its exceptional sensitivity and specificity for detecting pathogen genetic material.[1] However, limitations of PCR include the requirement for complicated heating−cooling cycles and the sensitivity to impurities present in the target nucleic acid preparations. Loop-mediated isothermal amplification (LAMP) and its variant for targeting RNA rather than DNA, Reverse Transcription (RT)-LAMP has become a popular alternative to PCR due to its one-pot nature, single temperature (isothermal) cycle, tolerance to impurities, and fast reaction times.[2−6] Compared to other detection methods, RT-LAMP offers rapid results, a low limit of detection, and high specificity.[7] LAMP shows promise, particularly in low-resource settings, where it can be configured into relatively low-cost point-of-care and simple field-based diagnostic tests. LAMP reactions can be visualized using both colorimetric and fluorescent methods, enabling observation with the naked eye[8,9] or through straightforward and low-cost optical systems.[10,11]

Fluidic and membrane-based devices have further promise for detecting pathogens in low-resource settings, including bedside and field-based diagnostics.[12] The development of suitable analytical platforms allows for timely, low-cost, and widespread testing. Recent developments in LAMP have integrated LAMP reactions into portable devices that streamline the detection process for field-based applications.[13] Developing LAMP reactions that can be performed on membranes and other nonstandard formats further removes design constraints and complications when designing LAMP-based fluidics,[14−19] reducing the need for fluid handling components.

Reverse transcriptase RT-LAMP assays targeting Severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2) (SCV2) were swiftly developed in response to the Coronavirus disease 2019 (COVID-19) pandemic, where researcher teams mobilized to expedite the design, development, optimization, and validation of RT-LAMP procedures.[20−23] The rapid development extended beyond assay development to include the creation of novel point-of-care diagnostic systems.[24−27] These tests are characterized by their speed, simplicity, and multiple detection methods available for LAMP amplicons.

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Despite this increased focus on RT-LAMP and other emerging diagnostic platforms, the stringent regulatory landscape for approving tests for use in direct human diagnostics impedes the rapid deployment of innovative diagnostic technologies.[28] In contrast, environmental sensing has a less burdensome approval process than human diagnostics, providing an attractive market for new technologies. Adapting LAMP for environmental sensing is an appealing proposition that may lead to the quick and widespread adoption of the technology, ultimately leading to further developments benefiting public health. For instance, the COVID-19 pandemic has illustrated the utility of environmental sensing, such as wastewater analysis for population-level public health surveillance.[29]

COVID-19 has also highlighted the critical role aerosols can play in pathogen transmission. SCV2 is infectious not only through direct contact and respiratory droplets but also via aerosols and can remain airborne for extended periods and travel further distances than larger respiratory particles.[30,31] Studies measuring SCV2 aerosol load typically have discrete capture, process, and detection steps with manual labor-intensive processes between and during each step.[32−34] Thus, detecting airborne SCV2 is often separated by time and distance from the sampling point, limiting the detection method's utility in informing decisions. As such, there is a need for the quick and straightforward detection of SCV2 in air samples for early outbreak identification, enabling the prompt implementation of infection control measures and targeted enhancements to the designed environment. Airborne virus detectors have been flagged as a priority Covid-19 research area through stakeholder engagement workshops.[35]

Here, by optimizing an RT-LAMP reaction to work directly on aerosol capture filters, we greatly simplify the process of detecting SCV2 in aerosols and provide an approach that can readily be incorporated into a microfluidic device. In this work, we formulate an RT-LAMP assay for use directly on an aerosol capture filter using a design of experiments (DOE) approach. DOE allows for efficient and statistically robust experiments, increasing the design space that can be explored and decreasing the time and effort required with common trial-and-error approaches to find an optimized formula. First, we determined additives that improved the detection time, and then we determined the optimal concentration of the additives. Using commercially available consumables, RT-LAMP reagents, and a fluorescence plate reader (as a prelude to designing and building customized equipment and materials), we demonstrated a sensitive and specific on-filter RT-LAMP reaction that outperforms the same reaction run in a conventional, microtube format. This methodology is an advance for detecting SCV2 directly from air and further enables rapid in situ detection of other airborne pathogens through the selection of suitable LAMP primers.

MATERIALS AND METHODS

The reagents used in these studies include WarmStart RT-Lamp Kit (E1700, New England BioLabs (NEB)) containing the master mix and fluorescent dye, Tween 20 (Sigma-Aldrich), tris hydrochloride (Sigma-Aldrich) (Tris-HCl), ethylenediaminetetraacetic acid (Mallinckrodt AR) (EDTA), dimethyl sulfoxide (Merck) (DMSO), magnesium sulfate (Sigma-Aldrich), polyethylene glycol (Lancaster, 200 MW) (PEG 200), ammonium sulfate (Chemsupply), trimethylglycine (Sigms-Aldrich) (Betaine), trehalose (Sigma-Aldrich), paraffin wax (ChemSupply), and bovine serum albumin (heat shock fraction, pH 7) (Sigma-Aldrich) (BSA). Primers were obtained in desalted form from Sigma-Aldrich.

Several brands of filter membranes were examined for the RT-LAMP reaction, including Whatman GF/C (glass fiber), Whatman QMA (quartz fiber), Whatman #1 (cellulose), Whatman #4 (cellulose), Gelman A/E (glass fiber), and ProSciTech MCE (mixed cellulose esters). Filters were cut to a final size with a 6 mm histology punch. Reactions in the plate reader were conducted in 96-well plates (Costar 3599 or 3603, Corning) sealed with adhesive tape (Microseal B, BioRad). Reactions in the OptiGene Genie HT were conducted in 8-tube Genie Strips.

On-filter RT-LAMP measurements were performed on a plate reader (CLARIOstar, BMG Labtech). The plate reader settings were the incubator set to 65 °C, fluorescence mode with fluorescein presets, bottom optic with a focal height of 3.6 mm, gain set to enhanced dynamic range, reading every 15 s with 10 flashes per reading. Total measurement times were 40 min for the limit of detection and specificity testing and 90 min for other tests. A positive result was indicated by an increase in fluorescence above a defined threshold, recorded as time-to-positive (Tp), expressed in decimal minutes. Data processing was performed in the instrument software (CLARIOstar MARS, BMG Labtech).

Up to 30 reactions were run at once in the 96-well plate, in a square from the B7 to G11 wells. This left an empty row around the perimeter to limit the risk of amplicon escape, kept reactions to the right side of the plate which had better heating, and allowed quick setup times reducing the time reactions were at room temperature before the heating cycle.

Liquid reactions and positive and negative controls were performed in an isothermal amplification instrument (Genie HT, OptiGene). Reactions were conducted at 65 °C with 30 min reaction times with readings every 30 s. A positive result was indicated by an increase in fluorescence at an emission wavelength of 540 nm (5(6)-carboxyfluorescein (FAM) channel) above a defined threshold, recorded as time to positive (Tp) expressed in decimal minutes.

Master mix and reactions were prepared in a separate biological safety cabinet from that used for template addition. Statistical analysis including the design of the DOE experiments was done by using a statistical software package (Minitab 20, Minitab LLC).

Cell Culture for SARS-CoV-2. Vero cells (within 30 passages from the original American Type Culture Collection [ATCC] stock) were maintained in Minimal Essential Media (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 10 μM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES), 2 mM glutamine, and antibiotics. Cell cultures were maintained at 37 °C in a 5% CO_2 incubator. All virus infection cultures were conducted within the high containment facilities of a physical containment level 3 (PC3) laboratory at the Doherty Institute. To generate stocks of SCV2, confluent Vero cell monolayers were washed once with MEM without FBS (infection media) and then infected with a known amount of SCV2 virus originally isolated from a patient.[36] After 1 h incubation at 37 °C in a 5% CO_2 incubator to enable virus binding, infection media containing 1 μg mL^{-1} *N*-tosyl-l-phenylalanine chloromethyl ketone-treated trypsin (TPCK-trypsin) was added, and flasks were returned to the incubator. After 3 days of incubation and microscopic confirmation of widespread cytopathic effect

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Table 1. Sequences of the RT-LAMP Primers Used in This Study

primer	final (1×) concentration	sequence
spike-F3	0.2 μM	ATTCTAAGCACACGCCTAT
spike-B3	0.2 μM	GAAGATAACCCACATAATAAGCT
spike-F1P	1.6 μM	ACCTATTGGCAAATCTACCAATGGTTTAGTGCGTGATCTCCCT
spike-B1P	1.6 μM	ATCACTAGGTTTCAAACTTTACTTGCCTGTCCAACCTGAAGAAGA
spike-LPF	0.4 μM	TTCTAAAGCCGAAAAACCCTG
spike-LPB	0.4 μM	CATAGAAG[T/G]TATTTGACTCCTGGTG

[] indicates mixed bases at that location.

(CPE), the supernatants were harvested, cell debris was pelleted by centrifugation at 2000*g* for 5 min, and then aliquots of the clarified supernatant were aliquoted and stored at −80 °C, to assess infectious SCV2 titers, both 50% tissue culture infectious dose ($TCID_{50}$). Briefly, serial dilutions of the stock virus were added to washed monolayers of Vero cells. After 1 h incubation to allow virus to adhere, for the $TCID_{50}$ assay, infection media containing 1 μg mL^{-1} TPCK-Trypsin was added. After 3 days of incubation, the dilution of stock required to cause CPE in at least 50% of wells ($TCID_{50}$) was determined via back calculation of microscopic confirmation of CPE in wells for a given dilution, using the Reed and Muench method.[37] Stocks of SCV2 used in this study had a $TCID_{50}$ of $10^{6.1}$ mL^{-1}. To inactivate the virus, 200 μL of neat stock was heated to 60 °C for 30 min and then cooled. Inactivation was confirmed via a complete lack of CPE using the $TCID_{50}$ assay.

Quantitative Real-Time PCR. As an approximation of the total amount of virus in the heat-inactivated preparation above, we used TaqMan quantitative PCR (Luna Universal Probe One-Step RT-qPCR Kit, NEB E3006) to estimate the number of E-gene copies in a sample as described.[38] The standard curves of E-gene copy number and titered heat-inactivated virus stocks were calculated (Figure S1, SI).

RT-LAMP Primers. The RT-LAMP primer sequences used (Table 1) were adapted from the literature.[39] Primers were mixed into a 10 or 12.5× stock that was used in experiments. Primer concentrations were those recommended by the NEB.

Filter Blocking and Heat Treatment. Filters with BSA or Tween 20 blocking were soaked in a BSA or Tween 20 solution (0.1% Tween 20 or 0.5, 1, or 2% BSA in water) for 1 h, pat dried with a paper towel, and then dried overnight. Final size (6 mm diameter) filters were cut from a larger filter after blocking and drying using a histology punch. This method was adapted from the literature.[40] Filter heat treatment involved heating Whatman GF/C filters in a furnace (SEM high-temperature oven) at 300 °C for 1 h with a 20 °C min^{-1} ramp.

Filter Selection and Wetting Level Experiments. Each RT-LAMP reaction contained master mix (NEB E1700 RT-LAMP kit, 12.5 μL, 2×), fluorescent dye (NEB E1700 RT-LAMP kit, 0.5 μL, 50×), primer solution (2.5 μL, 10×), stock solution of custom additives (2 μL, to give a final concentration of 0.1% Tween 20, 2 mM Tris-HCl, and 0.2 mM EDTA), water (6.5 μL), and inactivated SCV2 stock (10× dilution, 1 μL). Negative controls contained water in place of the SCV2 stock. Filters (6 mm circles) were loaded into a 96-well plate. The reaction mixture was made in a common pot, an aliquot (25 μL for filter selection experiments) was added to each filter, the plate was sealed with adhesive film, and the plate was heated and read using the plate reader. Positive and negative control samples were analyzed concurrent with the plate reader runs in a conventional LAMP instrument. Each filter was tested in triplicate. Reactions in the plate reader were manually determined by looking for the characteristic LAMP sigmoidal curve shape. Tp was recorded as the time when the trace passed over 450,000 relative fluorescence units.

Screening Additives Factorial DOE. The two-level factorial DOE experiment was designed with statistical software (Minitab 20). The DOE was split into two blocks, with different additives tested in each block. Each RT-LAMP reaction contained master mix (12.5 μL, 2×), dye (0.5 μL, 50×), primer solution (2.5 μL, 10×), additive stock solution (1 μL, to give a final concentration of 0.05% Tween 20, 1 mM Tris-HCl, and 0.1 mM EDTA), inactivated SCV2 stock (10× dilution, 1 μL), DOE additives as directed by the DOE (1 μL of each, 0−6 μL total), and water (balance to 25 μL). Negative nontemplate controls contained water in place of SCV2 stock.

20 μL of each reaction mixture was aliquoted onto each 6 mm filter disk in a 96-well plate. Reactions with paraffin had 2 pellets of solid paraffin (approximately 50 mg) added to the reaction well. The plate was sealed with adhesive film, and the plate was measured in the plate reader. Reactions that had not become positive after 90 min had a Tp of "90" recorded so they were not excluded from the analysis. Positive and negative control samples (25 μL) were analyzed at the same time in a conventional LAMP instrument.

Optimization Box−Behnken DOE. The four-factor Box−Behnken DOE experiment was designed with statistical software (Minitab 20). The experiment was performed in triplicate with blocking for each repeat. An identical second run of each condition was performed but without an RNA template to give a time to false positive (Tfp) measurement.

Each RT-LAMP reaction was constituted as described above, with 20 μL of each reaction mixture aliquoted onto each 6 mm filter disk (blocked filters were prepared with 1 or 2% BSA solutions as directed in the DOE) in a 96-well plate. The plate was sealed with adhesive film, and the reactions were monitored in the plate reader. As above, a positive and negative control sample (25 μL volume) were analyzed at the same time in a conventional LAMP instrument and on-filter reactions that had not become positive after 90 min had a time to positive (Tp) of "90" recorded so they were not excluded from the analysis.

Limit-of-Detection (LOD) Testing. Heat-treated SCV2 (4 μL, 10× dilution series) was spiked onto BSA-blocked filters (Whatman GF/C, 6 mm disks) sitting in a 96-well plate. Reaction solution (16 μL) containing master mix, dye, primers, and EDTA was then added to the spiked filters. Negative nontemplate controls contained water in place of SCV2 stock. The plate was sealed with an adhesive film and measured on the plate reader. The LOD was determined as the last dilution where 3/3 reactions recorded positive results.

Biological Specificity. Stocks of different heat-treated pathogens (4 μL) were spiked onto BSA-blocked filters (Whatman GF/C, 6 mm disks). The reaction solution (16

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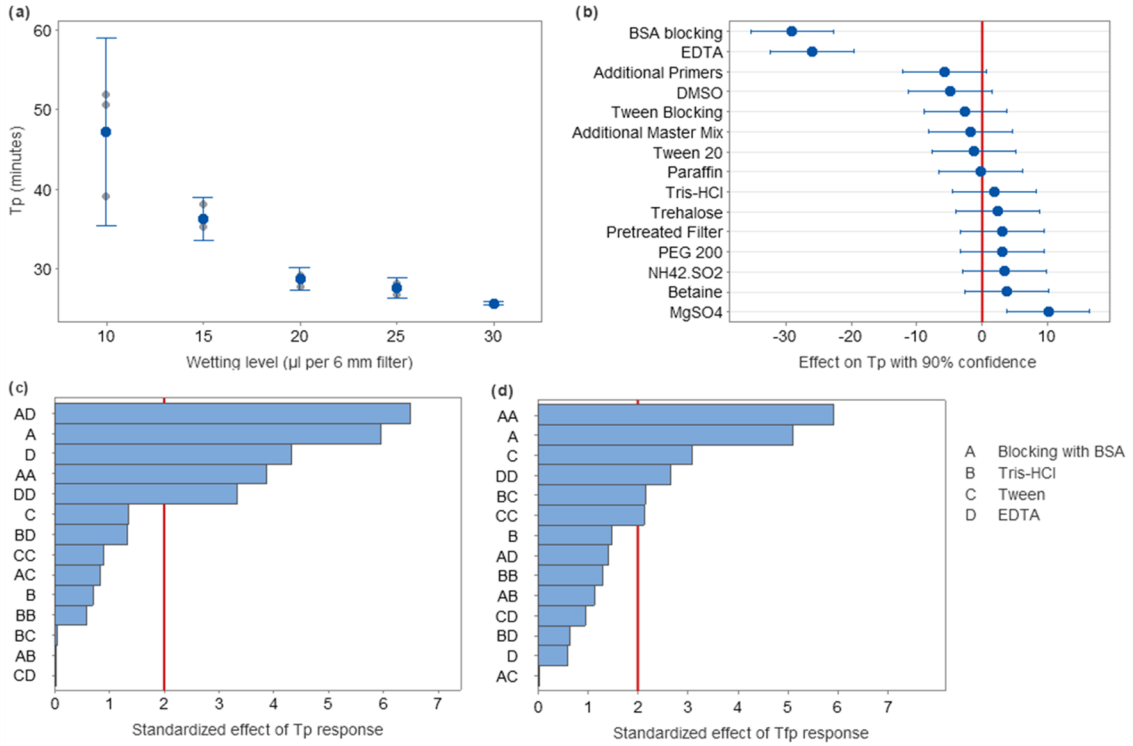

Figure 1. (a) Effect of reaction volume on reaction times for nonoptimized RT-LAMP reactions on-filter, showing individual values and 90% confidence intervals. (b) Plot showing the effect of different additives on RT-LAMP Tp from the first stage DOE. Error bars depict 90% confidence intervals. Confidence intervals that pass over the zero mark are not considered statistically significant. A negative value indicates that the additive reduced the Tp. (c, d) Pareto charts of standard effects of different additives on their effect on Tp and time to false positive (Tfp), respectively, at 95% confidence. Bars indicate the size but not the direction of the standard effect for the coded additives. The red horizontal line at 1.96 indicates the 95% significant level. Pairs of letters indicate interactions between terms and second-order coefficients.

μL) containing master mix, dye, primers, and EDTA was then deposited onto the spiked filters. Negative nontemplate controls contained water in place of pathogen stocks. The plate was sealed with adhesive film, and measurement was made using the plate reader.

RESULTS

Filter Selection. Our first task was to select an assay format for repeated filter LAMP optimization experiments. For convenience, we used a 96-well cell culture flat-bottom plate with filters cut to a 6 mm diameter to fit within each well while being sufficiently large to reliably detect fluorescence from the NEB E1700 RT-LAMP reaction format (see the Materials and Methods section) using the plate reader. A nonoptimized RT-LAMP formula containing a buffer composed of Tween 20, EDTA, and Tris-HCL (TET) was tested, as TET is commonly used as an extraction buffer with LAMP reactions. To identify a filter composition compatible with RT-LAMP, a series of reactions were run in the presence of different filters with a defined concentration of SCV2 RNA template. These tests showed that only the Whatman QMA and Whatman GF/C filters yielded positive RT-LAMP reactions (Table S1, SI). Out of the other filters tested, Whatman #1 and Whatman #4 cellulose filters and the mixed cellulose ester filters produced atypical amplification curves. This suggested that there may have been an interaction between cellulose filters and the dye. The Gelman A/E filters had a high background fluorescence signal and no easily discernible characteristic sigmodal LAMP shape. GF/C filters were chosen for subsequent experiments as they produce similar reaction times to the QMA filters, are less expensive, and were not subject to the same supply issues as the latter. Our observations therefore suggest that glass fiber filters perform well with RT-LAMP and are consistent with trials of filters and other isothermal amplification techniques.[41]

Finding Optimal Reaction Volumes for On-Filter Reactions. Different levels of the reaction mixture were tested on GF/C filters to determine the minimum volume of RT-LAMP reagents that could be used without compromising the assay performance. At 5 μL per 6 mm filter, reactions were not successful, with no increase in fluorescence signal above the threshold value observed within 90 min. While RT-LAMP reactions were reliable above 10 μL per filter, 20 μL per 6 mm filter (70.7 μL cm^{-2}) was chosen as the best compromise between minimizing both the reaction volume and the Tp (Figure 1a). This wetting amount was used in subsequent RT-LAMP reactions.

Finding Optimal Wetted Filter Composition. A factorial design of experiments (DOE) study was conducted to screen 16 potential additives for their impact on RT-LAMP reaction times at two concentrations (Table S2, SI). The factorial DOE framework enabled a more efficient and systematic evaluation of the additives' effects compared to assessing each factor individually. A fractional factorial design was utilized to reduce the number of runs and quickly identify the additives that significantly influenced the reaction times. Compared to earlier experiments, half the concentration of the additives Tween 20, Tris-HCl, and EDTA was used in all reactions. These additives were added to minimize the number of tests that did not produce a positive result in a 90 min reaction window, while having headroom for additives to

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Table 2. Optimized Formulas

formula	EDTA Level	BSA concentration	predicted Tp	95% confidence interval	predicted Tfp	95% confidence interval
1	0.4 mM	0.5%	22.5	11.6, 33.4	85.5	69.4, 101.6
2	0.1 mM	2%	22.0	12.2, 31.9	75.1	59.2, 91.0

Table 3. LOD Testing of the Liquid Reaction and Optimized Formulas

			liquid reaction			formula 1			formula 2		
stock dilution	$TCID_{50}$/mL	E-genes per reaction	positives	mean Tp (min)	std dev	positives	mean Tp (min)	std dev	positives	mean Tp (min)	std dev
1.0×10^{-01}	1.3×10^{5}	2.16×10^{7}	3/3	15.9	0.1	5/5	22.4	0.6	5/5	25.3	1.5
1.0×10^{-02}	1.3×10^{4}	2.16×10^{6}	3/3	18.8	0.3	5/5	24.3	0.5	5/5	26.6	1.0
1.0×10^{-03}	1300	2.16×10^{5}	3/3	21.6	0.3	5/5	26.3	0.7	5/5	27.1	3.4
1.0×10^{-04}	130	1.80×10^{4}	1/3	25.8	*	5/5	31.1	5.0	5/5	28.1	1.7
2.0×10^{-04}	63	9000	*	*	*	5/5	31.5	1.8	5/5	28.7	2.0
5.0×10^{-04}	**25**	3600	*	*	*	**5/5**	**32.7**	**1.2**	**3/5**	**36.1**	**3.5**
1.0×10^{-05}	13	1800	0/3	*	*	4/5	34.9	4.3	3/5	33.5	5.7
1.0×10^{-06}	1.3	180	0/3	*	*	1/5	36.3	*	0/5	*	*
no template	0	0	0/3	*	*	0/5	*	*	0/5	*	*

The LOD of the optimized formula is given in bold.

improve reaction times. All RT-LAMP reactions contained master mix, primers, and 8 mM $MgSO_4$ contained in the master mix. The testing of these additives in the reaction was performed by adding additional material to the standard reaction amounts. The additives were divided into two blocks because the available formulation volume could not simultaneously accommodate the testing of all additives. Assays that did not become positive had a Tp recorded as 90 min, so they were included in the analysis. A 90% significance level was used in the analysis. This lower significance level was used, as the experiment lacked repetitions, and it was deemed acceptable to include a nonsignificant factor in subsequent rounds of experiments.

The effects of the different additives on the Tp of the reaction at 90% confidence are shown in Figure 1b and tabulated (Table S3, SI). Of the 15 additives tested, only BSA blocking and EDTA significantly reduced reaction times, while $MgSO_4$ significantly increased reaction times.

The factors that significantly improved the reaction time, BSA blocking and EDTA, were carried over to a three-level Box–Behnken DOE study as well as Tris-HCl and Tween 20, which did not show as significant in the factorial experiment but were included in all runs, so their absence may be significant. Box–Behnken designs are a type of response surface methodology that do not include embedded factorial designs. Each factor is tested at three levels without requiring all factors to be tested simultaneously at their highest and lowest levels. This design allows for the efficient estimation of both first- and second-order effects with fewer experimental runs compared with other DOE methodologies. The levels of each reagent tested are shown in Table S4, SI. Each test was run with SCV2 RNA to give the Tp and without RNA to give the time to false positive (Tfp). This was done to assess whether additives made the RT-LAMP reaction more susceptible to false positives. The effects of the additives on Tp and Tfp are shown (Figure 1c,1d) and tabulated (Table S5, SI).

The *response optimizer* function in Minitab was used to find formulas that gave short Tp and long Tfp. Two formulas were selected for further testing, as shown in Table 2.

Optimized On-Filter RT-LAMP Limit of Detection. We used a stock of SCV2 carefully calibrated by qPCR to compare the limit of detection (LOD) of the two optimized formulas for on-filter RT-LAMP with conventional RT-LAMP (Table 3). The on-filter formula demonstrated comparable LODs, but both outperformed the conventional RT-LAMP, with a 50-fold increase in performance, with formula 1 reliably detecting SCV2 0.10 $TCID_{50}$ (3600 E-gene copies) per reaction.

Optimized On-Filter RT-LAMP Specificity. To ensure that the reaction was specific to SCV2, the reaction was run with nontarget pathogen samples (Table 4). No false positives or cross-reactivity was seen, showing that the RT-LAMP reaction remained specific when run on-filter.

Table 4. Testing of Nontarget Pathogens

pathogen	expected result	formula 1	formula 2
SARS-CoV-2	positive	positive	positive
Streptomyces lividans	negative	negative	negative
Mycobacterium marinum	negative	negative	negative
Mycobacterium abscessus	negative	negative	negative
Mycobacterium tuberculosis	negative	negative	negative
Mycobacterium ulcerans	negative	negative	negative
no template control (water blank)	negative	negative	negative

DISCUSSION

We systematically tested conditions that permitted a high-performance RT-LAMP reaction to run on a membrane filter. In doing so, we demonstrate that blocking GF/C glass fiber filters with BSA and incorporating EDTA into the formulation permit the use of an RT-LAMP assay on air collection filters without a loss in performance compared with the conventional liquid reaction format. Our on-filter RT-LAMP assay demonstrated improved sensitivity compared to its liquid form, achieving an LOD of 25 $TCID_{50}$/mL. While this LOD aligns with some studies,[20] it is higher than others that report LODs of sub-100 target RNA molecules per μL.[42,43] Using alternative primer sets and modifying enzyme type or composition for on-filter LAMP reactions are anticipated to further improve detection sensitivity.

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This work provides a proof-of-concept basis for the potential implementation of ambient air detection and person-wearable devices that can indicate the presence of airborne pathogens in an automated way. Given the potential for the filter material to impact reaction kinetics, including via adsorption, diffusion limitations, and reactions, and given the time-sensitive nature of airborne detection, determining the ideal reaction composition is paramount. We accordingly used a DOE framework to guide our research plan, which allowed us to explore a large design space efficiently while ensuring statistical robustness. First, we screened 15 different additives and conditions to assess whether they influenced the Tp. Subsequently, we optimized the concentrations of the four additives that were revealed to have the largest impact on Tp, yielding two optimized formulas that were tested for specificity. The DOE framework used here contrasts with one-factor-at-a-time experiments, which would have either required more experiments to gain the same insights or explored the design space with less robustness, potentially missing interaction effects and resulting in a less optimal solution.

This research aimed to demonstrate that RT-LAMP assays could be efficiently conducted directly with on-air capture filters with minimal sample preparation required. This is an important step in the development of all-in-one biological aerosol capture and detection devices. The ability to run the RT-LAMP assay directly on the aerosol filter simplifies the design architecture required, ultimately leading to a simpler, cheaper, more efficient, and robust design. The ability of RT-LAMP to detect pathogens without the need for RNA extraction or sample workup was an important consideration for its selection as the detection mechanism for our final goal of having a biological air sampling and detection device. This work will provide a starting point for other microfluidic devices to be developed based on LAMP or RT-LAMP detection mechanisms.

A wetting level of 20 μL per 6 mm filter was found to be the minimum wetness level without sacrificing reaction times. At this level of wetting, the filters are close to their "field capacity" of wetness, but not sodden or immersed in liquid. Below this level, reaction kinetics slow as the surface area of filter per volume of reaction increases, binding proportionally more reaction components to the filter and inhibiting the mixing of the reagent. BSA and EDTA were found to be significant additives for reducing the level of Tp. The proposed mechanism for this is that they reduce the interactions and binding between the template, reaction components, and the filter. Blocking with BSA prebinds BSA to binding sites on the filter, providing steric hindrance to reaction components that may otherwise bind to the sites. EDTA removes cations from the reaction solution, Mg^{2+} is the dominant cation in the reaction mixture at a concentration of 8 mM. Mg^{2+} is a cofactor for the polymerase enzyme and so is essential to the reaction. However, it also masks negatively charged groups on reaction components, reducing their solubility and increasing their binding to the filter. The addition of 0.4 mM EDTA to the liquid reaction resulted in quicker reaction times but did not result in improved sensitivity (Table S6, SI). If higher levels of EDTA were used, eventually the concentration of free magnesium ions would be expected to fall to a level where the polymerase enzyme functionality is reduced, though this was not observed with the range of concentrations utilized. In other master mixes that contain less magnesium sulfate, the effect of EDTA on the reaction is expected to be reduced. In general, a reaction solution with minimal ion concentration is ideal for running RT-LAMP reactions on a filter. This matches with common lab processes in which pure water is used as an elution buffer for nucleic acids off glass fiber filters.

Our study has some limitations. First, the Box−Behnken results showed "Blocking with BSA" and "EDTA" squared terms as significant. This indicates that a parabola was fit to the data. This is likely an overfitting to the underlying dataset that contains only 3 levels. Box−Behnken designs are more suited to the linear range of the variables. Future experiments should reduce the difference between high and low values or use another method, such as a response surface model with five values for each parameter. While this would likely give neater results, it is unlikely that the conclusions would differ significantly from the Box−Behnken design in this instance.

In this work, we used liquid spikes of lab-cultured heat-treated SCV2 deposited onto the filters as a simulant for collecting aerosolized virus by air filtration. This allowed accurate and reproducible dosing of the filters without any risk of infection. This is a compromise over using infectious virus, aerosolizing virus in the lab, or sampling air in the vicinity of COVID patients capable of transmitting the virus. These other approaches have disadvantages such as presenting a risk of infection, having limited or uncontrolled dosing of filters, requiring higher biological control measures, or requiring ethics approval. Due to the excellent aerosol collection efficiency of Whatman GF/C filters of over 99.6%[44] and the fragility of SCV2, it is predicted that the assay will maintain sensitivity when used with infectious aerosolized virus in droplets from expectorate materials and/or within fomites.

Being able to perform multiple reactions on-filter at once on standard lab equipment and consumables allowed this research to proceed promptly, without requiring bespoke equipment to be designed and made. The experimental method for conducting on-filter RT-LAMP measurements is shown (Figure S2, SI). The standard lab consumables gave a high degree of confidence in the sealing of the reactions and the containment of amplicons. The optics of the plate reader were able to easily detect the change in fluorescence of the reactions; detection through the bottom of the plates removed any issues of condensation in the optical path creating noise in the measurement. The plate reader did, however, have nonoptimum heating. The incubator of the plate reader has a maximum temperature of 65 °C, which is our target temperature. Unlike a conventional isothermal incubator where the reaction chamber is surrounded by a heating block with direct contact, in the plate reader, the 96-well plate is heated by air convection with no direct contact with a heat block. A 96-well plate has a thermal mass much higher than that of the plasticware used in a conventional format LAMP reaction. This leads to reactions having a much slower temperature ramp rate than that of reactions in a conventional LAMP incubator. The effect of the slow heating can be seen in the traces from the plate reader (Figure S3, SI). In the initial stages of RT-LAMP reactions, as the temperature of the dye in the reaction increases, fluorescence decreases. In the plate reader, this takes approximately 8 min; in the isothermal amplification instrument, this heating occurs in less than 1 min.

This slow heating affects reaction times and would be expected to decrease specificity as there is a higher chance of nonspecific amplification occurring when LAMP reactions are below their optimal temperature, and it may be the cause of the 50-fold increase in sensitivity we observed for on-filter

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reactions compared to the liquid reactions. The reverse transcriptase used in the master mix (WarmStart RTx Reverse Transcriptase, NEB) is inhibited below 40 °C and has an optimal reaction temperature of 55−65 °C. The slower heating profile for reactions in the plate reader gives the reverse transcriptase additional time to make the initial cDNA copies. Other possible explanations for the increased sensitivity of on-filter RT-LAMP include BSA improving sensitivity for on-filter measurements while not being present in the liquid reactions or the high-surface-area membrane creating positive surface effects. To further investigate the cause of the improved sensitivity in the plate reader measurements, DNA targets could be tested, which eliminate the reverse transcription step. Additionally, custom heating profiles with slower ramp times could be investigated in the isothermal amplifier, providing further insights.

This work lays the groundwork for designing an all-in-one device capable of running a complete RT-LAMP workflow on collected biological aerosol samples. While this work has focused on SCV2, the flexibility of LAMP allows an easy pathway to other pathogens. Such a device is a significant engineering challenge that will require a sampling pump, heating blocks, and optics systems for heating and measuring the RT-LAMP reaction. Naked eye detection of the LAMP reaction could be achieved by employing colorimetric, fluorescent, or lateral flow assays for end point determination, simplifying the optical setup required.

A final device will also require the components to direct airflow, for storing and then releasing reagents, and for sealing the reaction vessel to contain the LAMP amplicons. Such a solution will address the limitations discussed above and have a quicker heating ramp than was possible in the plate reader used in this work.

CONCLUSIONS

A design of experiments methodology was used to optimize reaction additives to run RT-LAMP assays for SCV2 detection directly on aerosol collection filters. The assay was optimized for reaction speed, and the final formula showed improved sensitivity over the same RT-LAMP master mix and primers run in a conventional format, without loss of specificity. The observed reaction speeds (Tp) were limited by the general equipment (plate reader) used, which had a nonoptimal heating profile. This research shows the viability of running a sensitive and specific molecular detection assay directly on an aerosol collection filter and thus lays the foundation for developing portable, autonomous devices that detect airborne pathogens.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05784.

Method for comparing liquid reactions with and without EDTA (Tables S1−S6) (Figures S1−S3) (PDF)

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J.F., N.J.F., and T.P.S. conceived the study. J.F., J.Y.H.L., J.L.M., J.L.P., and I.R.M. conducted experiments. J.F. and T.P.S. wrote the manuscript. D.J.C., S.T.M., J.L.M., and J.Y.H.L. edited the manuscript. All authors read and approved the final draft of the manuscript.

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Notes

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■ ABBREVIATIONS

ABI	Applied Biosystems instruments
B1P	backward inner primer
B3	backward outer primer
BSA	bovine serum albumin
CPE	cytopathic effect
COVID-19	Coronavirus disease 2019
DMSO	dimethyl sulfoxide
DOE	design of experiments
E-gene	viral envelope protein gene
EDTA	ethylenediaminetetraacetic acid
F1P	forward inner primer
F3	forward outer primer
FAM	5(6)-carboxyfluorescein
FBS	fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
L15	Leibovitz-15
LAMP	loop-mediated isothermal amplification
LOD	limit-of-detection
LPB	loop backward primer
LPF	loop forward primer
MEM	minimal essential media
NEB	New England BioLabs
PC3	physical containment level 3
PCR	polymerase chain reaction
RT-LAMP	reverse transcription loop-mediated isothermal amplification
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SCV2	severe acute respiratory syndrome Coronavirus 2 (SARS-CoV-2)
$TCID_{50}$	tissue culture infective dose 50
TET	tris-EDTA-tween buffer
TPCK-trypsin	*N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin
Tp	time to positive
Tfp	time to false positive

■ REFERENCES

(1) Filchakova, O.; Dossym, D.; Ilyas, A.; Kuanysheva, T.; Abdizhamil, A.; Bukasov, R. Review of COVID-19 Testing and Diagnostic Methods. *Talanta* **2022**, *244*, No. 123409.

(2) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**, *28* (12), No. e63.

(3) Nagamine, K.; Hase, T.; Notomi, T. Accelerated Reaction by Loop-Mediated Isothermal Amplification Using Loop Primers. *Mol. Cell. Probes* **2002**, *16* (3), 223−229.

(4) Howson, E. L. A.; Kurosaki, Y.; Yasuda, J.; Takahashi, M.; Goto, H.; Gray, A. R.; Mioulet, V.; King, D. P.; Fowler, V. L. Defining the Relative Performance of Isothermal Assays That Can Be Used for Rapid and Sensitive Detection of Foot-and-Mouth Disease Virus. *J. Virol. Methods* **2017**, *249*, 102−110.

(5) Parida, M.; Posadas, G.; Inoue, S.; Hasebe, F.; Morita, K. Real-Time Reverse Transcription Loop-Mediated Isothermal Amplification for Rapid Detection of West Nile Virus. *J. Clin. Microbiol.* **2004**, *42* (1), 257−263.

(6) Fukuta, S.; Iida, T.; Mizukami, Y.; Ishida, A.; Ueda, J.; Kanbe, M.; Ishimoto, Y. Detection of Japanese Yam Mosaic Virus by RT-LAMP. *Arch. Virol.* **2003**, *148* (9), 1713−1720.

(7) Alhamid, G.; Tombuloglu, H.; Rabaan, A. A.; Al-Suhaimi, E. SARS-CoV-2 Detection Methods: A Comprehensive Review. *Saudi J. Biol. Sci.* **2022**, *29* (11), No. 103465.

(8) Tomita, N.; Mori, Y.; Kanda, H.; Notomi, T. Loop-Mediated Isothermal Amplification (LAMP) of Gene Sequences and Simple Visual Detection of Products. *Nat. Protoc.* **2008**, *3* (5), 877−882.

(9) Deng, H.; Jayawardena, A.; Chan, J.; Tan, S. M.; Alan, T.; Kwan, P. An Ultra-Portable, Self-Contained Point-of-Care Nucleic Acid Amplification Test for Diagnosis of Active COVID-19 Infection. *Sci. Rep.* **2021**, *11* (1), No. 15176.

(10) Buultjens, A. H.; Vandelannoote, K.; Sharkey, L. K.; Howden, B. P.; Monk, I. R.; Lee, J. Y. H.; Stinear, T. P. Low-Cost, Open-Source Device for High-Performance Fluorescence Detection of Isothermal Nucleic Acid Amplification Reactions. *ACS Biomater. Sci. Eng.* **2021**, *7* (10), 4982−4990.

(11) Kaarj, K.; Akarapipad, P.; Yoon, J.-Y. Simpler, Faster, and Sensitive Zika Virus Assay Using Smartphone Detection of Loop-Mediated Isothermal Amplification on Paper Microfluidic Chips. *Sci. Rep.* **2018**, *8*, No. 12438.

(12) Xia, Y.; Si, J.; Li, Z. Fabrication Techniques for Microfluidic Paper-Based Analytical Devices and Their Applications for Biological Testing: A Review. *Biosens. Bioelectron.* **2016**, *77*, 774−789.

(13) Chang, C.-C.; Chen, C.-C.; Wei, S.-C.; Lu, H.-H.; Liang, Y.-H.; Lin, C.-W. Diagnostic Devices for Isothermal Nucleic Acid Amplification. *Sensors* **2012**, *12* (6), 8319−8337.

(14) Choi, J. R.; Hu, J.; Tang, R.; Gong, Y.; Feng, S.; Ren, H.; Wen, T.; Li, X.; Abas, W. A. B. W.; Pingguan-Murphy, B.; Xu, F. An Integrated Paper-Based Sample-to-Answer Biosensor for Nucleic Acid Testing at the Point of Care. *Lab Chip* **2016**, *16* (3), 611−621.

(15) Jawla, J.; Kumar, R. R.; Mendiratta, S. K.; Agarwal, R. K.; Kumari, S.; Saxena, V.; Kumar, D.; Singh, P.; Boby, N.; Rana, P. Paper-Based Loop-Mediated Isothermal Amplification and Lateral Flow (LAMP-LF) Assay for Identification of Tissues of Cattle Origin. *Anal. Chim. Acta* **2021**, *1150*, No. 338220.

(16) Yang, Z.; Xu, G.; Reboud, J.; Ali, S. A.; Kaur, G.; McGiven, J.; Boby, N.; Gupta, P. K.; Chaudhuri, P.; Cooper, J. M. Rapid Veterinary Diagnosis of Bovine Reproductive Infectious Diseases from Semen Using Paper-Origami DNA Microfluidics. *ACS Sens.* **2018**, *3* (2), 403−409.

(17) Jiang, X.; Loeb, J. C.; Pan, M.; Tilly, T. B.; Eiguren-Fernandez, A.; Lednicky, J. A.; Wu, C.-Y.; Fan, Z. H. Integration of Sample Preparation with RNA-Amplification in a Hand-Held Device for Airborne Virus Detection. *Anal. Chim. Acta* **2021**, *1165*, No. 338542.

(18) Reboud, J.; Xu, G.; Garrett, A.; Adriko, M.; Yang, Z.; Tukahebwa, E. M.; Rowell, C.; Cooper, J. M. Paper-Based Microfluidics for DNA Diagnostics of Malaria in Low Resource Underserved Rural Communities. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116* (11), 4834−4842.

(19) Seok, Y.; Joung, H.-A.; Byun, J.-Y.; Jeon, H.-S.; Shin, S. J.; Kim, S.; Shin, Y.-B.; Han, H. S.; Kim, M.-G. A Paper-Based Device for Performing Loop-Mediated Isothermal Amplification with Real-Time Simultaneous Detection of Multiple DNA Targets. *Theranostics* **2017**, *7* (8), 2220−2230.

(20) Lee, J. Y. H.; Best, N.; McAuley, J.; Porter, J. L.; Seemann, T.; Schultz, M. B.; Sait, M.; Orlando, N.; Mercoulia, K.; Ballard, S. A.; Druce, J.; Tran, T.; Catton, M. G.; Pryor, M. J.; Cui, H. L.; Luttick, A.; McDonald, S.; Greenhalgh, A.; Kwong, J. C.; Sherry, N. L.; Graham, M.; Hoang, T.; Herisse, M.; Pidot, S. J.; Williamson, D. A.; Howden, B. P.; Monk, I. R.; Stinear, T. P. Validation of a Single-Step, Single-Tube Reverse Transcription Loop-Mediated Isothermal Amplification Assay for Rapid Detection of SARS-CoV-2 RNA. *J. Med. Microbiol.* **2020**, *69*, 1169.

(21) Fowler, V. L.; Armson, B.; Gonzales, J. L.; Wise, E. L.; Howson, E. L. A.; Vincent-Mistiaen, Z.; Fouch, S.; Maltby, C. J.; Grippon, S.;

https://doi.org/10.1021/acsomega.4c05784
ACS Omega 2024, 9, 40832−40840

Munro, S.; Jones, L.; Holmes, T.; Tillyer, C.; Elwell, J.; Sowood, A.; de Peyer, O.; Dixon, S.; Hatcher, T.; Patrick, H.; Laxman, S.; Walsh, C.; Andreou, M.; Morant, N.; Clark, D.; Moore, N.; Houghton, R.; Cortes, N. J.; Kidd, S. P. A Highly Effective Reverse-Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) Assay for the Rapid Detection of SARS-CoV-2 Infection. *J. Infect.* **2021**, *82* (1), 117−125.

(22) Lu, R.; Wu, X.; Wan, Z.; Li, Y.; Jin, X.; Zhang, C. A Novel Reverse Transcription Loop-Mediated Isothermal Amplification Method for Rapid Detection of SARS-CoV-2. *Int. J. Mol. Sci.* **2020**, *21* (8), 2826.

(23) Baek, Y. H.; Um, J.; Antigua, K. J. C.; Park, J.-H.; Kim, Y.; Oh, S.; Kim, Y.; Choi, W.-S.; Kim, S. G.; Jeong, J. H.; Chin, B. S.; Nicolas, H. D. G.; Ahn, J.-Y.; Shin, K. S.; Choi, Y. K.; Park, J.-S.; Song, M.-S. Development of a Reverse Transcription-Loop-Mediated Isothermal Amplification as a Rapid Early-Detection Method for Novel SARS-CoV-2. *Emerging Microbes Infect.* **2020**, *9* (1), 998−1007.

(24) Garneret, P.; Coz, E.; Martin, E.; Manuguerra, J.-C.; Brient-Litzler, E.; Enouf, V.; Obando, D. F. G.; Olivo-Marin, J.-C.; Monti, F.; van der Werf, S.; Vanhomwegen, J.; Tabeling, P. Performing Point-of-Care Molecular Testing for SARS-CoV-2 with RNA Extraction and Isothermal Amplification. *PLoS One* **2021**, *16* (1), No. e0243712.

(25) Donia, A.; Furqan Shahid, M.; Hassan, S.; Shahid, R.; Ahmad, A.; Javed, A.; Nawaz, M.; Yaqub, T.; Bokhari, H. Integration of RT-LAMP and Microfluidic Technology for Detection of SARS-CoV-2 in Wastewater as an Advanced Point-of-Care Platform. *Food Environ. Virol.* **2022**, *14* (4), 364−373.

(26) Malic, L.; Brassard, D.; Fonte, D. D.; Nassif, C.; Mounier, M.; Ponton, A.; Geissler, M.; Shiu, M.; Morton, K. J.; Veres, T. Automated Sample-to-Answer Centrifugal Microfluidic System for Rapid Molecular Diagnostics of SARS-CoV-2. *Lab Chip* **2022**, *22* (17), 3157−3171.

(27) Ganguli, A.; Mostafa, A.; Berger, J.; Aydin, M. Y.; Sun, F.; de Ramirez, S. A. S.; Valera, E.; Cunningham, B. T.; King, W. P.; Bashir, R. Rapid Isothermal Amplification and Portable Detection System for SARS-CoV-2. *Proc. Natl. Acad. Sci. U.S.A.* **2020**, *117* (37), 22727−22735.

(28) Ivanov, A. Barriers to the Introduction of New Medical Diagnostic Tests. *Lab. Med.* **2013**, *44* (4), e132−e136.

(29) Shah, S.; Gwee, S. X. W.; Ng, J. Q. X.; Lau, N.; Koh, J.; Pang, J. Wastewater Surveillance to Infer COVID-19 Transmission: A Systematic Review. *Sci. Total Environ.* **2022**, *804*, No. 150060.

(30) Tang, S.; Mao, Y.; Jones, R. M.; Tan, Q.; Ji, J. S.; Li, N.; Shen, J.; Lv, Y.; Pan, L.; Ding, P.; Wang, X.; Wang, Y.; MacIntyre, C. R.; Shi, X. Aerosol Transmission of SARS-CoV-2? Evidence, Prevention and Control. *Environ. Int.* **2020**, *144*, No. 106039.

(31) Morawska, L.; Cao, J. Airborne Transmission of SARS-CoV-2: The World Should Face the Reality. *Environ. Int.* **2020**, *139*, No. 105730.

(32) Lednicky, J. A.; Shankar, S. N.; Elbadry, M. A.; Gibson, J. C.; Alam, M. M.; Stephenson, C. J.; Eiguren-Fernandez, A.; Morris, J. G.; Mavian, C. N.; Salemi, M.; Clugston, J. R.; Wu, C.-Y. Collection of SARS-CoV-2 Virus from the Air of a Clinic Within a University Student Health Care Center and Analyses of the Viral Genomic Sequence. *Aerosol Air Qual. Res.* **2020**, *20* (6), 1167−1171.

(33) Mallach, G.; Kasloff, S. B.; Kovesi, T.; Kumar, A.; Kulka, R.; Krishnan, J.; Robert, B.; McGuinty, M.; den Otter-Moore, S.; Yazji, B.; Cutts, T. Aerosol SARS-CoV-2 in Hospitals and Long-Term Care Homes during the COVID-19 Pandemic. *PLoS One* **2021**, *16* (9), No. e0258151.

(34) Chia, P. Y.; Coleman, K. K.; Tan, Y. K.; Ong, S. W. X.; Gum, M.; Lau, S. K.; Lim, X. F.; Lim, A. S.; Sutjipto, S.; Lee, P. H.; Son, T. T.; Young, B. E.; Milton, D. K.; Gray, G. C.; Schuster, S.; Barkham, T.; De, P. P.; Vasoo, S.; Chan, M.; Ang, B. S. P.; Tan, B. H.; Leo, Y.-S.; Ng, O.-T.; Wong, M. S. Y.; Marimuthu, K. Singapore 2019 Novel Coronavirus Outbreak Research Team. Detection of Air and Surface Contamination by SARS-CoV-2 in Hospital Rooms of Infected Patients. *Nat. Commun.* **2020**, *11* (1), No. 2800.

(35) Tong, A.; Sorrell, T. C.; Black, A. J.; Caillaud, C.; Chrzanowski, W.; Li, E.; Martinez-Martin, D.; McEwan, A.; Wang, R.; Motion, A.; Bedoya, A. C.; Huang, J.; Azizi, L.; Eggleton, B. J. Research Priorities for COVID-19 Sensor Technology. *Nat. Biotechnol.* **2021**, *39* (2), 144−147.

(36) Caly, L.; Druce, J.; Roberts, J.; Bond, K.; Tran, T.; Kostecki, R.; Yoga, Y.; Naughton, W.; Taiaroa, G.; Seemann, T.; Schultz, M. B.; Howden, B. P.; Korman, T. M.; Lewin, S. R.; Williamson, D. A.; Catton, M. G. Isolation and Rapid Sharing of the 2019 Novel Coronavirus (SARS-CoV-2) from the First Patient Diagnosed with COVID-19 in Australia. *Med. J. Aust.* **2020**, *212* (10), 459−462.

(37) Reed, L. J.; Muench, H. A Simple method of estimating fifty per cent endpoints12. *Am. J. Epidemiol.* **1938**, *27* (3), 493−497.

(38) Vandelannoote, K.; Buultjens, A. H.; Li, L.; Sharkey, L. K.; Herisse, M.; Pidot, S. J.; Hoang, T.; Howden, B. P.; Monk, I. R.; Seemann, T.; Lee, J. Y. H.; Stinear, T. P. Accessible Platform for High-Throughput COVID-19 Molecular Diagnostics and Genome Sequencing Using a Repurposed 3D Printer for RNA Extraction. *ACS Biomater. Sci. Eng.* **2021**, *7* (9), 4669−4676.

(39) Mohon, A. N.; Oberding, L.; Hundt, J.; van Marle, G.; Pabbaraju, K.; Berenger, B. M.; Lisboa, L.; Griener, T.; Czub, M.; Doolan, C.; Servellita, V.; Chiu, C. Y.; Greninger, A. L.; Jerome, K. R.; Pillai, D. R. Optimization and Clinical Validation of Dual-Target RT-LAMP for SARS-CoV-2. *J. Virol. Methods* **2020**, *286*, No. 113972.

(40) Shetty, P.; Ghosh, D.; Singh, M.; Tripathi, A.; Paul, D. Rapid Amplification of *Mycobacterium tuberculosis* DNA on a Paper Substrate. *RSC Adv.* **2016**, *6* (61), 56205−56212.

(41) Rohrman, B. A.; Richards-Kortum, R. R. A Paper and Plastic Device for Performing Recombinase Polymerase Amplification of HIV DNA. *Lab Chip* **2012**, *12* (17), 3082−3088.

(42) Marino, F. E.; Proffitt, E.; Joseph, E.; Manoharan, A. A Rapid, Specific, Extraction-Less, and Cost-Effective RT-LAMP Test for the Detection of SARS-CoV-2 in Clinical Specimens. *PLoS One* **2022**, *17* (4), No. e0266703.

(43) Janíková, M.; Hodosy, J.; Boor, P.; Klempa, B.; Celec, P. Loop-Mediated Isothermal Amplification for the Detection of SARS-CoV-2 in Saliva. *Microb. Biotechnol.* **2021**, *14* (1), 307−316.

(44) Baron, P. A.; Willeke, K. *Aerosol Measurement: Principles, Techniques, and Applications*, 2nd ed.; Wiley-Interscience: Hoboken, N.J, 2005.

https://doi.org/10.1021/acsomega.4c05784
ACS Omega 2024, 9, 40832−40840