Paper microfluidic device enables rapid and on-site wastewater surveillance in community settings

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Article

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Abstract

Tracking genomic sequences as microbial biomarkers in wastewater has been used to determine community prevalence of infectious diseases, contributing to public health surveillance programs. Here we report upon a low-cost, rapid, and user-friendly paper microfluidic platform for SARS-CoV-2 and influenza detection, using a loop-mediated isothermal amplification (LAMP), with the signal read simply using a mobile phone camera. Sample-to-answer results were collected in < 1.5 hours providing rapid detection of SARS-CoV-2 and influenza viruses in wastewater, with a detection limit of < 20 copies μL^{-1}. The device was subsequently used for on-site testing of SARS-CoV-2 in wastewater samples from four quarantine hotels at London Heathrow Airport, showing comparable results to those obtained using a gold-standard polymerase chain reaction assay, as reference. Our sensing platform, which enables rapid and localized wastewater surveillance and does not require the sample to be sent to a centralized laboratory, is potentially an important public health tool for a wide variety of future applications, in community settings.

Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), brought about significant public health and socioeconomic challenges[1]. In mild cases, the symptoms of the disease are similar to a number of other respiratory infections. The Centers for Disease Control and Prevention (CDC) emphasized the importance of distinguishing between infections caused by SARS-CoV-2 and influenza viruses[2]. The SARS-CoV-2 virus not only causes respiratory illness, but is associated with gastrointestinal infections, with viral material being excreted in feces (and surviving within sewage for several days). Due to the highly infectious nature of SARS-CoV-2, reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays have been used to confirm the presence of SARS-CoV-2 in wastewater, within community settings[3].

Wastewater surveillance has previously been shown to be capable of detecting a wide range of infectious diseases[4], including those caused by the SARS-CoV-2 virus[5], providing readily available aggregated samples. Although surveillance techniques have been proposed as an early warning tool to identify diseases within communities, a current limitation of their widespread use is the local availability of diagnostic centers, with appropriately trained staff. Currently samples are therefore sent to centralized laboratories, with associated lengthy times for results (typically 24–48 hours). Notwithstanding these delays, wastewater surveillance was conducted across 45 sewage sites in the UK, demonstrating that wastewater-based epidemiology (WBE) can detect SARS-CoV-2 signal, 4–5 days in advance of the emergence of clinical cases[6].

Existing methods used for WBE analysis of SARS-CoV-2 involve either PCR amplification assays (as a gold standard analytical method) and/or genome sequencing for the identification of new variants[7]. For example, deep sequencing of 94 urban water catchments was used to infer the spatiotemporal

abundance of predefined variants of SARS-CoV-2 from a large number of wastewater samples[8]. However, the long analysis time, the low viral loads (often also diluted by rainwater run-off in the wastewater) as well as nucleic acid degradation and the presence of PCR inhibitors in sewage, all currently present significant analytical challenges.

As an alternative to PCR, isothermal amplification methods such as loop-mediated isothermal amplification (LAMP) have attracted increasing attention for the detection of a range of pathogens[9]. Amplification of RNA viral biomarkers can be easily integrated, in the form of reverse transcription loop-mediated isothermal amplification (RT-LAMP), to allow amplification for the detection of SARS-CoV-2[10] as well as influenza A[11] and influenza B[12]. However, currently many LAMP-based detection methods may still rely upon centralized laboratories, with costly instruments and well-trained technicians, preventing their more widespread use in point-of-care (POC) diagnostics.

Paper-based microdevicees have been used to integrate nucleic acid extraction, purification, elution, amplification, and detection into affordable and easy to use tests[13]. The whole analysis process can be completed by folding the paper device in different ways without fixed power supplies. With advances in microfluidic technology, LAMP and RT-LAMP based assays have also been implemented on paper-based lateral flow devices, enabling the detection of a variety of pathogens[14], with an aim of providing point-of-need diagnostics that are affordable, sensitive, specific, user-friendly, rapid, robust, equipment-free, and deliverable to end-users (ASSURED assays), which when including real-time connectivity and ease of specimen collection have come to be known as REASSURED assays[15].

We have contributed to the development of paper-based devices for POC diagnostics, demonstrating the multiplexed determination of microbial species from whole blood, using the paper-origami technique to enable DNA extraction, LAMP and array-based fluorescence detection[16]. We then further developed this paper-origami device for the detection of three bovine infectious reproductive diseases in semen samples from rural India[17]. Recently, we used paper origami technology to perform multiplex detection of malaria field testing in Uganda, Africa[18], demonstrating that paper-based devices can be integrated into field-ready modules and provide rapid, sensitive, and specific detection capabilities comparable to laboratory-based instruments.

During the COVID-19 pandemic, paper-based devices can be used as an alternative to RT-qPCR without the need for laboratory instruments or specialized technicians[19]. The combination of paper microfluidic devices and WBE also provides new insights into SARS-CoV-2 detection to monitor disease transmission. For example, a paper device based on clustered regularly interspaced short palindromic repeats (CRISPR)/Cas12a and RT-LAMP was proposed for the detection of N, E and S genes in wastewater[20], with a detection limit of 10–310 copies mL^{-1}, tested using wastewater spiked with genomic sequences and/or viruses.

Thus, the concept of the potential SARS-CoV-2 testing in sewage has also been described, to trace the source of COVID-19 and determine the presence of SARS-CoV-2 carriers in a community[21]. Using portable paper microfluidic devices, community testing would enable sewage to be analyzed with lower testing costs and higher testing efficiency. Early warning sensing systems based on WBE, and paper microfluidic devices are expected to track the source of SARS-CoV-2 and help monitor and control the COVID-19 pandemic. To achieve this, we now describe now a paper microfluidic device, using RT-LAMP assay for rapid detection of SARS-CoV-2 and influenza A/B in wastewater, collected from community settings, as a new tool for WBE. The method comprised a simple hand-held syringe-based sample preparation system to enrich pathogen samples from wastewater, enabling the extraction, purification, amplification, and detection all to take place in community settings. Results were read by illumination using a hand-held UV torch and/or collected by a mobile-phone camera (to enable quantification).

The paper microfluidic device was first optimized using a model surrogate virus porcine reproductive and respiratory syndrome virus (PRRSV) in order to define channel geometries and sensing area sizes. To assess the sensitivity and specificity of the paper-based platform for pathogen detection, transcribed RNA at different concentrations was spiked into water to construct mimic real samples for laboratory testing. Subsequently, using this optimized paper-based platform, we tested 20 wastewater samples collected by Anglian Water (using a laboratory-based RT-qPCR amplification assay as a “gold-standard” reference)

Finally, the optimized paper microfluidic devices were demonstrated for the local analysis of wastewater from four quarantine hotels based in London Heathrow Airport, showing its significant promise for rapid and on-site wastewater surveillance for public health. The technique not only shows the value of the methodology for localized community testing in well-resourced environments, but also illustrates the potential application for the paper microfluidic device in the resource-limited settings, e.g., in low- and middle-income countries where there is an increasing need to develop rapid, portable, low-cost, and user-friendly platforms to detect pathogens.

Results

During the COVID-19 outbreak in 2020–2022, the spread of the pandemic was monitored and mitigated by designing detection platforms to distinguish viruses in wastewater[3]. In general, these systems only provided infection trends, with the effectiveness of the methods being limited by samples having to be transported to centralized laboratories for analysis (and data generated was retrospective and not “real-time”). We chose to overcome these limitations by developing a paper-based lateral flow test for wastewater epidemiology, measuring nucleic acid biomarkers in situ, at the point-of-sampling.

We first optimized the paper microfluidic device using PRRSV as a model surrogate virus, **Figure S1**. The general testing scheme is shown in Fig. 1. and subsequently confirmed the validity of the developed device for virus detection in wastewater.

Real-time And Paper-based Lamp Assay For Virus Detection

The nucleic acids released into wastewater from SARS-CoV-2 are RNA sequences/fragments, and the strategy for their amplification and detection involved performing reverse transcriptase (RT) production of cDNA, prior to the LAMP. As stated, our assays were designed based upon three well-established target biomarker genes (GenBank: MN908947), the locations of which are shown in Fig. 2a. The ORF1ab LAMP primers were directed against a 203 nt long sequence from nucleotide positions 13434 to 13636 of the ORF1ab gene. The S LAMP primers were spanning a 245 nt long sequence from nucleotide positions 23693 to 23937 of the S gene. The N LAMP primers covered a 217 nt long sequence from nucleotide positions 28525 to 28741 of the N gene. The principle of LAMP assay, Fig. 2b is based upon using 4–6 different primers to identify 6–8 different regions on the target sequence which is amplified by DNA polymerase at a constant temperature. In addition, the use of loop primers is used to reduce amplification time[22]. The LAMP assay can amplify a small amount of target DNA, generating a large amount of amplified DNA in less than one hour.

Sensitivity And Specificity Of Real-time Rt-qpcr Assay On Viruses

10-fold serial dilutions (10^6 to 1 copies μL^{-1}) of in vitro transcribed viral RNA was prepared to evaluate the sensitivity of real-time RT-qPCR assay. In real-time qPCR assay, the reaction products of each cycle provide a fluorescent signal from the probe binding to the target sequence. The cycle threshold (C_t) is the number of cycles at which the intensity of the fluorescent signal exceeds the threshold signal for positivity[23]. C_t is a relative measurement of the target concentration in the sample, where lower C_t value represents higher target concentration. As illustrated in **Figure S2**, the ORF1ab RT-qPCR assay demonstrated a linear range of $10–10^6$ copies μL^{-1} with a LOD of 10 copies μL^{-1}. The RT-qPCR amplicons were then analyzed on 3% agarose gel for the presence of DNA bands. The detection limits for ORF1ab, S and N RNA were around 10 copies μL^{-1}. For real-time RT-qPCR assays of three influenza genotypes and associated agarose gel electrophoresis, similar trends were observed (**Figure S3**). Detection limits were as low as 10 copies μL^{-1} for H1N1, H7N9 and influenza B genotypes. The standard curve of the RT-qPCR assay was also used as a reference standard for the corresponding RT-LAMP assay.

Sensitivity And Specificity Of Real-time Rt-lamp Assay

The 10-fold serial dilutions (10^6 to 1 copies μL^{-1}) of in vitro transcribed SARS-CoV-2 viral RNA was prepared to evaluate the sensitivity of real-time RT-LAMP assay. The real-time RT-LAMP assay for the detection of viruses was performed on a QuantStudio 3 Real-Time PCR System. Calcein together with Mn^{2+} was used as a fluorescent metal indicator for RT-LAMP assay. As the RT-LAMP reaction proceeds, Mn^{2+} and newly-formed $P_2O_7^{4-}$ combined to form a $Mn_2P_2O_7$ precipitate, which leads to calcein with fluorescent signal. The concentration ratio of calcein to Mn^{2+} was optimized by real-time LAMP assay. Threshold time (T_t) is defined as the time corresponding to 10% of the maximum fluorescence intensity and is a function of target concentration. The T_t of the real-time LAMP assay is analogous to the C_t of the

real-time PCR assay. The optimal concentration ratio of calcein to Mn^{2+} was 1:20 (**Figure S4**), which was applied in subsequent LAMP experiments. The assay showed a LOD of 10 copies μL^{-1} for the detection of ORF1ab, S and N RNA (**Figure S5**). The LAMP amplicons were observed under ultraviolet light and then analyzed on 3% agarose gel for the presence of ladder pattern. The detection limits for H1N1, H7N9 and influenza B genotypes were also 10 copies μL^{-1} (**Figure S6**). In addition, our RT-LAMP assay showed good reproducibility for synthetic target RNA in triplicate.

As illustrated in **Figure S5**, the time required for positive detection with the ORF1ab primer set ranged from 25 minutes for 10^6 copies μL^{-1} to 35 minutes for 10 copies μL^{-1}, while for the S primer set, the time required for positive detection ranged from 20 min for 10^6 copies μL^{-1} to 30 min for 10 copies μL^{-1}. For the N primer set, the time required for positive detection ranged from 35 minutes for 10^6 copies μL^{-1} to 60 minutes for 10 copies μL^{-1}. The RT-LAMP assay based on these primers indicated a LOD of 10 copies μL^{-1} within 60 minutes, so the reaction time for RT-LAMP assay was set as 60 min. In contrast, the RT-qPCR assay achieved a LOD of 10 copies μL^{-1} within 2 h. Furthermore, the negative reaction solution showed no fluorescence when illuminated using a UV torch, while the positive reaction solution showed obvious fluorescence. The negative reaction solution remained light yellow when observed under sunlight, while the positive reaction solution appeared light green. The detection limit obtained via visual read-out was consistent with LOD obtained through analyzing real-time LAMP fluorescence amplification curves, indicating that the calcein-based RT-LAMP assay could be applied in subsequent paper-based tests.

The LOD for SARS-CoV-2 (10 copies μL^{-1}) was similar to or lower than a nanoparticle-based lateral flow biosensor integrated with multiplex RT-LAMP assay for ORF1ab detection (12 copies μL^{-1})[24], a RT-LAMP assay based on the Loopamp RNA amplification kit for S detection (100 copies μL^{-1})[25], and a colorimetric RT-LAMP assay based on the WarmStart Colorimetric RT-LAMP Master Mix for N detection (100 copies μL^{-1})[26]. In another study, a modified two-step RT-LAMP assay for SARS-CoV-2 detection[27], adding a reverse transcription incubation step (55°C, 10 min) prior to the RT-LAMP reaction, was reported to achieve a detection limit as low as 1 copy μL^{-1}. Thermal lysis (95℃, 10min) inactivated nucleases in crude samples, while the reverse transcription (55°C, 10min) improved annealing between RNA template and primers to allow efficient complementary strand synthesis. Our assay indicated a good detection limit compared with published reports, with a higher detection limit when compared to reverse-transcription droplet digital PCR (RT-ddPCR) assay[28].

Field-testing Wastewater With Paper-based Device

A general scheme for our paper-based RT-LAMP assay is shown in Fig. 3, with the wastewater sample being first filtered twice using syringe filters (0.45 μm and 0.025 μm) to remove impurities whilst retaining virus particles on the membrane filter. As illustrated in Fig. 3b schematically, fluorescent labels indicate different target-specific reactions. The results are the representative images for single (target 1), duplex (target 1–2), and triplex (target 1–3) target detection, together with internal negative control and internal

positive control to determine the effectiveness of the test. In the paper-based RT-LAMP assay, green color indicates a positive test result while no green color indicates a negative test result.

To analyze the feasibility of the paper-based platform to detect SARS-CoV-2 in real wastewater samples, we performed RT-LAMP assay on 20 wastewater samples collected by Anglian Water, and then compared the detection results with standard RT-qPCR detection results. Reference analysis involved a kit-based RNA extraction and RT-qPCR assay. The different relationships between C_t and date are presented in Fig. 4a-c, respectively. New cases by specimen date (between 20 October and 30 November 2020) are also shown in three figures. Lower C_t values are indicative of more new cases according to the specimen date.

The results demonstrated that among the 20 wastewater samples, 6 samples tested positive for ORF1ab gene, 17 samples tested positive for S gene, and 16 samples tested positive for N gene. It can be inferred that the detection efficiency of ORF1ab gene in wastewater was lower than S gene and N gene, which is consistent with a previous report[29]. The current detection of SARS-CoV-2 RNA mainly relies on the detection of ORF1ab and N genes. N protein provides a highly immunogenic and conserved amino acid sequence, indicating that N gene is a suitable target for monitoring SARS-CoV-2. In detail, it has also been found that N gene was the predominant positive fragment in COVID-19 patients, while ORF1ab gene presented a lower percentage of positives[30]. According to the observation of the dynamic changes of ORF1ab and N genes in COVID-19 patients, the positive duration of N gene is longer than that of ORF1ab.

Results for the wastewater samples were also tested on the paper-based RT-LAMP assay. The results of the paper-based platform for 20 wastewater samples, analysed in triplicate, are presented in Fig. 4d. For ORF1ab gene, 7 sample chambers showed obvious fluorescence, while no fluorescence was observed in the remaining 13 samples. For S and N genes, 16 sample chambers showed obvious fluorescence, while no fluorescence was observed in the remaining 4 samples. The results obtained by our platform were in good agreement with the laboratory-based RT-qPCR assay. Sixteen of the 17 RT-qPCR S-positive samples and all the RT-qPCR N-positive samples (n = 16) were detected as positive by the platform. Of the 17 RT-qPCR S-positive samples, only one sample was detected as negative. In addition, one sample tested by the platform showed a false-positive result for ORF1ab gene, indicating possible carry-over contamination of the LAMP amplicons. Contamination may be reduced by adding Uracil-DNA Glycosylase (UDG) and deoxyuridine triphosphate (dUTP) to the RT-LAMP reaction[31]. Overall, we demonstrate that the paper-based platform can be used for sensitive and specific detection of SARS-CoV-2 in wastewater.

To demonstrate the feasibility of the proposed platform for field detection, we used the paper-based device for on-site SARS-CoV-2 detection at a hotel near Heathrow Airport from 12 to 17 July 2021, with samples collected using industry-standard autosamplers. The wastewater samples were collected daily from 4 locations near the airport from Monday to Friday, over 5 days, Fig. 5. **Figure S7** presents the associated equipment, consumables and reagents for on-site SARS-CoV-2 detection, including wax-printed μPAD, syringe filter, heat blocker, and UV torch. A syringe-based system was designed to enrich

pathogen samples from wastewater (**Figure S8**), allowing for the subsequent extraction, purification, amplification, and detection (**Figure S9**). The enrichment step does not require any electricity or laboratory-based equipment, thus offering potential field testing in resource-limited areas. Nucleic acids were extracted from pathogens, amplified by RT-LAMP, and detected using fluorescent signal. Results were obtained using a hand-held UV torch and captured with a mobile phone camera.

Discussion

The proposed platform successfully detected the ORF1ab, S and N genes of SARS-CoV-2 in wastewater samples (Fig. 5). Laboratory-based RT-qPCR detection of SARS-CoV-2 was also performed on wastewater samples collected from 4 sites from Monday to Friday, including (f) Site 1, (g) Site 2, (h) Site 3 and (i) Site 4. The RT-LAMP results were largely consistent with the RT-qPCR results. The N gene presented the highest detection rate in wastewater samples, followed by the S and ORF1ab genes. Molecular diagnosis of SARS-CoV-2 currently relies on the detection of specific and conserved ORF1ab, S, N, RdRp and E genes. Despite the high specificity of ORF1ab gene, clinical applications suggest that ORF1ab gene is less sensitive than other genes[32]. ORF1ab gene shows less sensitivity than N, and S genes due to shorter sequence which is likely be degraded in the wastewater system. On the other hand, nucleotide variations are more frequent in ORF1ab gene than in N gene[33]. Nucleotide variations in the published ORF1ab-related qPCR primer sequences are also more frequent than in N gene.

With the emergence of emerging SARS-CoV-2 variants, the detection of N gene in wastewater is more stable than ORF1ab gene because N gene has fewer nucleotide variations. Higher abundance of sub-genomic N gene messenger RNAs also results in N gene being more sensitive than ORF1ab and S genes[34]. Wastewater samples collected from Site 4 showed the most positive results for ORF1ab gene, possibly due to the highest number of hotel guests or the highest infection rate among guests. In contrast, Site 2 wastewater samples presented the least number of positive tests for S gene, with one important possibility being that this may be due to dilution of viral load or a decrease in infection cases during testing. If community testing strategies are to be developed in the future, great care must be taken around the consideration of how the sample is collected, consistently within a given site or between sites. Two results from the paper-based device were inconsistent with RT-qPCR results, which may be due to a potential carry-over of inhibitors from syringe-based system and paper-based extraction in comparison to kit-base RNA extraction.

During testing, British authorities used hotels near Heathrow Airport to isolate travelers, including those who tested positive for COVID-19. This activity was consistent with the World Health Organization's advice, who identified WBE as a potential tool for COVID-19 monitoring. Our paper-based platform provides results within 1.5 hours, which is faster than the laboratory-based RT-qPCR method (24h or more). The platform demonstrated acceptable sensitivity and specificity, and does not require expensive equipment, making it suitable for field applications by non-specialists. This allows local and regional public health officials to take timely measures to reduce the spread of SARS-CoV-2. Future research will focus on integrating concentration, extraction, detection, and analysis modules into a single device to

standardize and automate wastewater monitoring to identify SARS-CoV-2 and other pathogens. When using WBE to assess virus transmission in a community, it is challenging to correlate the number of viruses with the actual number of cases. The decay rate of SARS-CoV-2 in wastewater, PCR inhibitors, and wastewater flow rates may affect the efficacy of the assay results[35]. Further effort is required to understand and further improve sampling, sample handling, target detection and result analysis.

In conclusion, the proposed microfluidic paper-based analytical device for rapid detection of pathogens in water enables sample-to-answer analysis in less than 1.5 hours. Despite the fact that the paper-based platform only provides a semi-quantitative result, it serves as a rapid, cost-effective, and user-friendly tool for community wastewater analysis, reducing the burden on pandemic surveillance and general diagnostic departments. Results can be read using a hand-held UV torch or collected by a mobile phone camera, which also might allow for result quantification. Our platform was able to detect viruses at < 20 copies RNA per reaction. Compared to gold-standard PCR assay, our paper-based platform provides similar or higher specificity and sensitivity for pathogen detection at a much cheaper and faster way, providing a high-resolution data set for highly responsive measurement during the pandemic. More importantly, it can be deployed in low-resource setting, for example, low- and middle-income countries to support an establishment of early warning system and has the potential for rapid monitoring of pathogens in water.

Materials And Methods

Materials

Whatman Grade 1 Qualitative Filter Paper was purchased from GE Healthcare Worldwide (UK). Microfluidic channels were wax printed by a Xerox ColorQube 8580 digital wax printer from Xerox (UK). Black Cast Acrylic was obtained from Stockline Plastics (UK) and was processed by a laser cutter from Laserscript (UK). A Bio-Rad C1000 Thermal Cycler, horizontal electrophoresis apparatus and a Gel Doc XR + Imager were from Bio-Rad Laboratories (UK). A hot plate, Digital Dry Bath, and UV torch (366 nm) were from Fisher Scientific (UK). A QuantStudio 3 Real-Time PCR System, TaqMan Fast Virus 1-Step Master Mix, Qubit 4 Fluorometer and MicroAmp Optical Adhesive Film were from Thermo Fisher Scientific (UK).

Brilliant III Ultra-Fast SYBR Green QPCR Master Mix was purchased from Agilent Technologies (UK), while GspSSD2.0 LF DNA Polymerase and AMV-Reverse Transcriptase were obtained from OptiGene (UK). EvaGreen was from Cambridge BioScience (UK), while calcein, manganese (II) chloride, syringes, syringe filters and Swinnex filter holders were from SIGMA (UK). Punchers were from Kai Europe GmbH (Germany). The RNeasy Mini Kit and Zymoclean Gel DNA Recovery Kit were from Qiagen (USA) and Zymo Research (Germany), respectively. The HiScribe T7 High Yield RNA Synthesis Kit and Monarch RNA Cleanup Kit were from New England Biolabs (UK). All primers, DNA fragments and plasmids were ordered from SIGMA (UK), Eurofins Genomics (Germany) and GENEWIZ (Germany).

Preparation Of Template Dna And Rna

Six sets of LAMP primers were selected for the ORF1ab[36], S[37] and N[38] genes on SARS-CoV-2, as well as H1N1[39], H7N9[40] and influenza B[41] genotypes. For initial optimization experiments, we designed 6 DNA fragments containing target sequences and primers with a T7 RNA polymerase promoter, Fig. 2. Details of these LAMP primers and PCR primers for in vitro transcription are provided in **Table S1 and Table S2**. Intact viral RNA was also extracted from samples using a RNeasy Mini Kit.

For in vitro transcribed RNA template preparation, the target sequence was first PCR-amplified with PCR primers containing the T7 RNA polymerase promoter. The PCR reaction was performed in a total volume of 20 µL, comprising 10 µL PCR Master Mix, 0.8 µL PCR primers (0.4 µm), 1 µL EvaGreen, 7.2 µL ddH2O and 1 µL DNA fragment (5 ng μL^{-1}). The PCR conditions consisted of an initial denaturation step at 95°C for 7 min, followed by 40 cycles, with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min 30 s. The PCR amplicons were analyzed on 1.5% agarose gel stained with SYBR Safe DNA gel stain, then extracted and purified from agarose gel using a Zymoclean Gel DNA Recovery Kit to form an in vitro transcription template. Thereafter, the purified PCR amplicons were incubated at 37°C for 2 hours to synthesize artificial RNA using the HiScribe T7 High Yield RNA Synthesis Kit. Next, the synthesized RNA was treated with DNase I to remove the DNA template and purified using a Monarch RNA Cleanup Kit. Finally, the purified RNA was stored at -80°C for further use.

For quantification of the template nucleic acid, the copy number of each template nucleic acid was determined by their molecular weight. The Qubit 4 Fluorometer with Qubit 1X dsDNA HS Assay Kit or Qubit RNA HS Assay Kit (Thermo Fisher Scientific, UK) was used to measure the concentration of the template DNA or RNA, the DNA/RNA Copy Number Calculator (http://endmemo.com/bio/dnacopynum.php) was then used to calculate the copy number of the template nucleic acid. The target sequence detected by LAMP was first amplified by real-time PCR with LAMP outer primers to confirm the accuracy of target detection. Subsequently, 10-fold serial dilutions of template nucleic acid were performed for sensitivity testing and to produce a standard curve.

Rt-qpcr Assay

The real-time RT-qPCR assay was performed on a QuantStudio 3 Real-Time PCR System. The qPCR primers sets for ORF1ab[42], S[43] and N[42] genes, as well as H1N1[44], H7N9[45] and influenza B[46] genotypes, porcine reproductive and respiratory syndrome virus (PRRSV)[47] and mitochondrial DNA (mtDNA)[48] are detailed in **Table S3**. mtDNA was chosen as the target of the internal positive control while ddH_2O was added to the internal negative control. All RT-qPCR reactions were performed in triplicate in a total volume of 20 µL, including 6 µL TaqMan Fast Virus 1-Step RT-PCR Master Mix, 1.25 µL PCR primers, 7.75 µL ddH2O and 5 µL sample. The RT-qPCR conditions consisted of a reverse transcription step at 50°C for 5 min, an initial denaturation step at 95°C for 20 s, followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and an extension at 72°C for 30 s.

Rt-lamp Assay

The real-time RT-LAMP assay was performed using a thermocycler. The LAMP primer sets for ORF1ab[36], S[37], N[38], H1N1[39], H7N9[40], FluB[41], PRRSV[49] and mtDNA[50] are detailed in **Table S1**. All RT-LAMP reactions were performed in triplicate in a total volume of 20 µL, including 10 µL LAMP Master Mix, 3 µL LAMP primers (0.1 µm F3/B3, 0.8 µm FIP/BIP, 0.6 µm LF/LB), 2 µL calcein (25 µM), 1 µL $MnCl_2$ (500 µM), 1 µL Gsp 2.0, 0.2 µL reverse transcriptase, 0.8 µL ddH2O and 2 µL sample. The RT-LAMP was carried out at a constant reaction temperature of 63℃ for 60 min. In addition, the RT-LAMP assay was performed in the digital dry bath. In an RT-LAMP assay, calcein was used as a colorimetric indicator[51]. Positive results can be determined visually from the color change of the reaction solution from yellow to green. Results can also be read by a hand-held UV torch or digitally collected using a mobile phone camera.

Design, Characterization, And Optimization Of Paper Device With High-throughput Detection

The paper device was first characterized with geometries optimized using a model pathogen, PRRSV, as a surrogate, see **Figure S1**. Subsequently, the design of the paper-based device for the detection of 3 targets was defined and is shown in Fig. 1a. The device contains three components, including a filter paper-based microfluidic device with wax-printed microfluidic channels, a plastic device sealed with a single-sided optical film and one glass fiber circular disc (4 mm in diameter) for absorbing nucleic acids from the sample. For the 5 reaction chambers in the plastic device, N represents the internal negative control, T1, T2 and T3 represent the targets, and P represents the internal positive control.

The unfolded paper device comprised a sample preparation zone and a detection zone and was mounted to a plastic plate to show the detection results. The paper unit for each panel had a footprint of 3 cm × 3 cm (3 × 24 cm when unfolded). The paper device was fabricated on the Whatman Grade 1 Qualitative Filter Paper in three steps (Fig. 1c). First, the structure of the device was designed in the software CorelDRAW, with factors such as operation convenience being taken into consideration during the design process. Then, the device was fabricated by printing hydrophobic wax on the filter paper, defining the microfluidic channels. Finally, the patterned filter paper was baked on a hot plate at 130°C for 5 min to melt the printed wax into the porous structure of the filter paper (preventing lateral flow by capillarity). The wax-penetrated filter paper was cut into individual devices for subsequent LAMP experiments.

Paper-based Device For Pathogen Detection

This work demonstrated a paper-based platform for multiplex detection of SARS-CoV-2 and influenza viruses in wastewater. **Figure S10** illustrates the workflow of the platform for virus detection in wastewater, including wastewater concentration, paper-based nucleic acid extraction, device-based RT-LAMP reaction, and signal read-out. In this case, a volume of wastewater was first filtered through syringe filters (0.45 µm and 0.025 µm), followed by the addition of lysis buffer, after which the filtrate was

incubated at room temperature for 10 minutes (the filter pores are large enough to allow the viruses to pass through[52]).

The paper device was assembled with a glass fiber within a 4 mm hole punched into the printed panel. The lysate was introduced onto the glass fiber, where nucleic acids were adsorbed (the excess liquid was absorbed by the hydrophilic disc present on the third panel of the paper device). Any debris, including cell membrane residues were rinsed off using washing buffer. The paper device was then folded for elution and nucleic acids were released from the glass fiber, reaching the printed hydrophilic channels. Circular spots, defined by punching, formed the detection zone, contained within sample chambers of the plastic plate (which also contained RT-LAMP reagents). An optical adhesive film was also used to cover the plastic plate to avoid reagent evaporation during isothermal amplification in a digital dry bath. Finally, the results were read using a hand-held UV torch and the fluorescence signal was captured using a mobile phone camera.

The whole sample-to-answer process took < 1.5 hours and only required basic experimental equipment and consumables (flasks and syringes). In contrast, the workflow for the reference methods, performed on wastewater samples, and involving ultrafiltration concentration, kit-based RNA extraction and RT-qPCR assay took ~ 4 hours. With the modification of operating procedures, process workflow and experimental reagents (e.g., change the pore size of the syringe filter or LAMP primers), this paper-based platform can be readily extended to detect other viruses and bacteria in various environmental water samples, establishing new avenues for low-cost, rapid, and user-friendly multiplex detection of pathogens.

Sars-cov-2 Detection In Hotels

Autosamplers were used for wastewater sampling in the four quarantine hotels around London Heathrow Airport in the summer of 2021. Sampling personnel were clothed in standard personal protective equipment (PPE) used for wastewater sampling, such as protective clothing, safety glasses, face masks, and gloves. The autosampler was programmed to collect composite wastewater samples (50 mL wastewater samples every 30 minutes) over a 24-hour period, i.e., the samples that flowed through the sampling point over a day. An aliquot of each sample collected by the autosampler was transferred to 1 L plastic bottles, stored in a thermostatic bag filled with ice packs for sample transport, and then transferred to the hotel basement for sample analysis, showing in **Figure S11**. The process for on-site SARS-CoV-2 detection is presented in the section Paper-based device for pathogen detection, and the analytical reagents and paper-based kits were transported to the testing hotel from Cranfield University.

Declarations

Contributions

Y.P. and Z.Y. conceived the study and designed the experiments. Y.P. performed the experiments with the supervision of ZY. J.C. provided support for the development and edited the mansurcipt. B.W. provided

suggestions and edited the manuscirpt. All authors took part in the interpretation of results and preparation of materials for the manuscript. Y.P. and Z.Y. wrote the manuscript with inputs from all co-authors. Z.Y. supervised and acquired the funding of the study.

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Figures

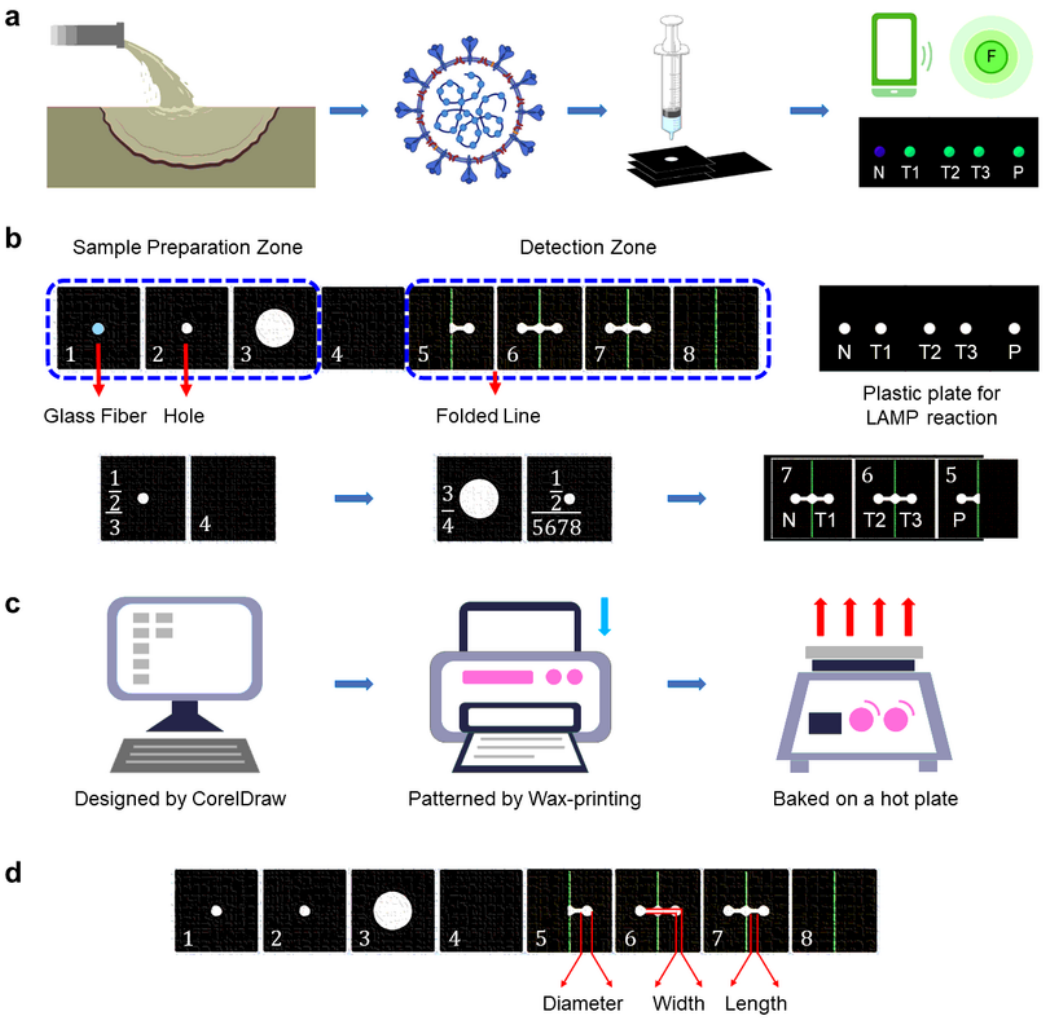

Figure 1

Design and characterisation of paper device with high-throughput detection. (a) Schematic showing the sample-to-answer diagnostic process for virus detection in wastewater using a paper-based device; (b) Design of the paper-based device. The device contains three components, comprising a filter paper-based microfluidic device, a plastic device sealed with a single-sided optical film and one glass fiber circular disc. N represents the internal negative control, T1, T2 and T3 represent the targets, and P represents the

internal positive control; (c) Fabrication of the paper-based device. The device was designed in CorelDraw, then patterned by wax-printing and finally baked on a hot plate; (d) Illustration of the paper device, indicating the specific dimensions - the pore size, channel width and channel length.

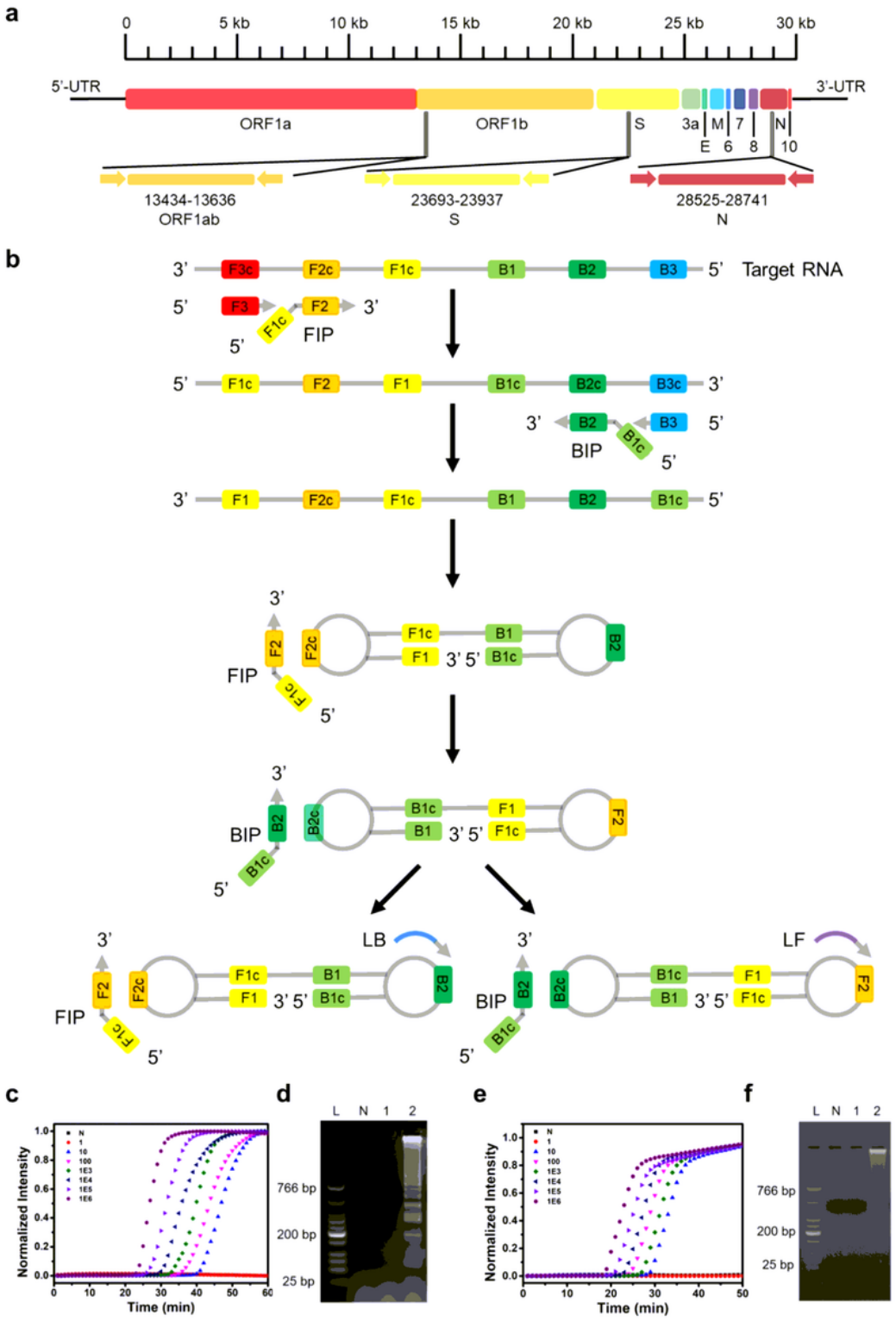

Figure 2

Design and optimization of LAMP assay for viral RNA detection (including SARS-CoV-2 and influenza) (a) Map showing LAMP primers for SARS-CoV-2 detection. Visualization of primers on the SARS-CoV-2 genome (GenBank: MN908947); (b) Principle of LAMP assay; (c) RT-LAMP assay for N gene; (d) Gel electrophoresis for N LAMP products (L: Ladder; -: ddH_2O; 0: 1 copy μL^{-1}; 1: 10 copies μL^{-1}); (e) RT-LAMP assay for influenza B genotype; (f) Gel electrophoresis for influenza B LAMP products (L: Ladder; -: ddH_2O; 0: 1 copy μL^{-1}; 1: 10 copies μL^{-1}).

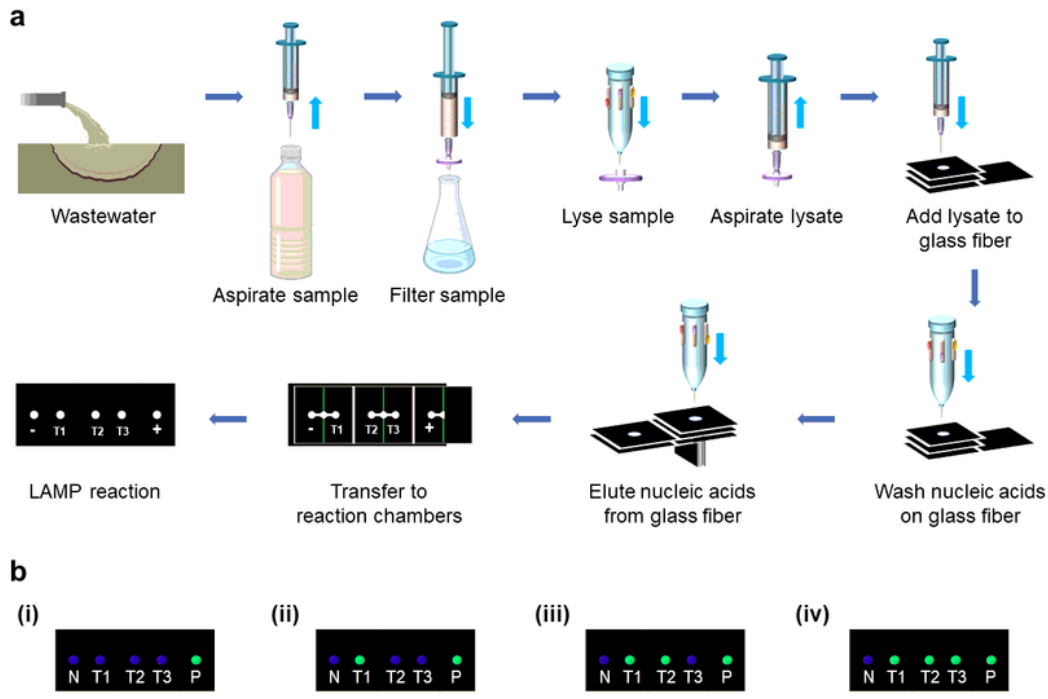

Figure 3

The on-site enrichment workflow and paper-based device for multiplexed detection of SARS-CoV-2. (a) Figure showing a schematic illutrating the analytical work-flow The water sample was first filtered twice, followed by sample lysis, expanding upon the schematic shown in Figure 1(a).. The lysate was introduced onto the paper device with a glass fibre frit, which was followed by two cycles of nucleic acid washing and elution. Subsequently, the paper discs on the paper devices were punched out and added to the sample chambers of a plastic plate for RT-LAMP reaction. The results were read using a hand-held UV torch or semi-quantitatively using a mobile phone camera (b) The labels indicate different target-specific reactions. N: internal negative control; P: internal positive control; T1: target 1; T2: target 2; T3: target 3. The results are the representative images for single (target 1), duplex (target 1-2), and triplex (target 1-3) target detection, together with negative control and positive control to determine the effectiveness of the test (green color for positive tests and no green color for negative tests).

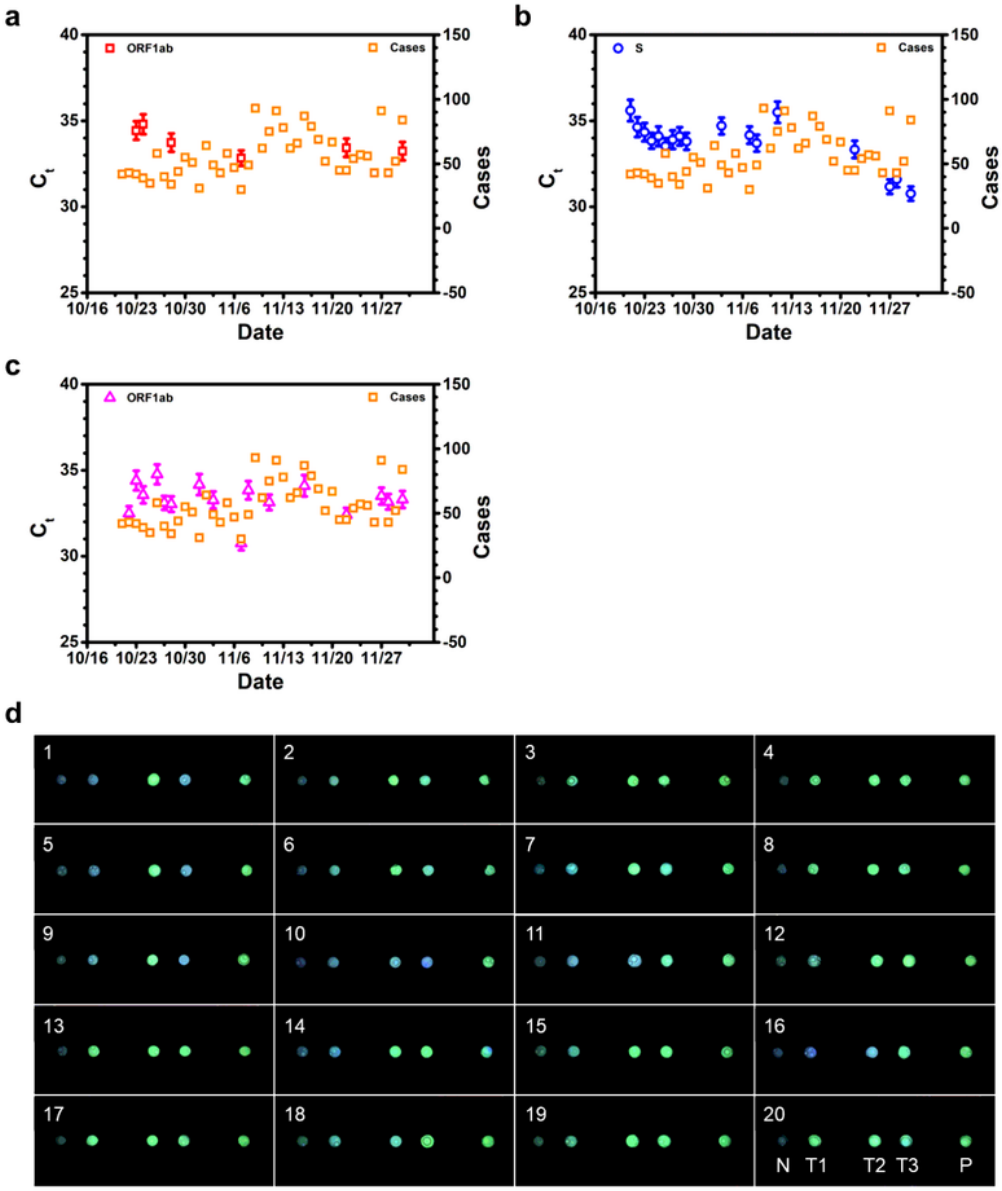

Figure 4

Testing of a cohort of wastewater samples in local WWTPs in 2020 RT-PCR testing of (a) ORF1ab gene, (b) S gene and N gene of SARS-CoV-2 collected from local WWTPs in November 2020 at Anglian Water Treatment plant with the confirmed positive data disclosed from local city council within the catchment of the populations; (d) Images of the paper-based RT-LAMP assay were illuminated with a hand-held UV

torch and captured by mobile phone camera for SARS-CoV-2 detection in wastewater. N: ddH_2O; T1: ORF1ab; T2: S; T3: N; P: mtDNA.

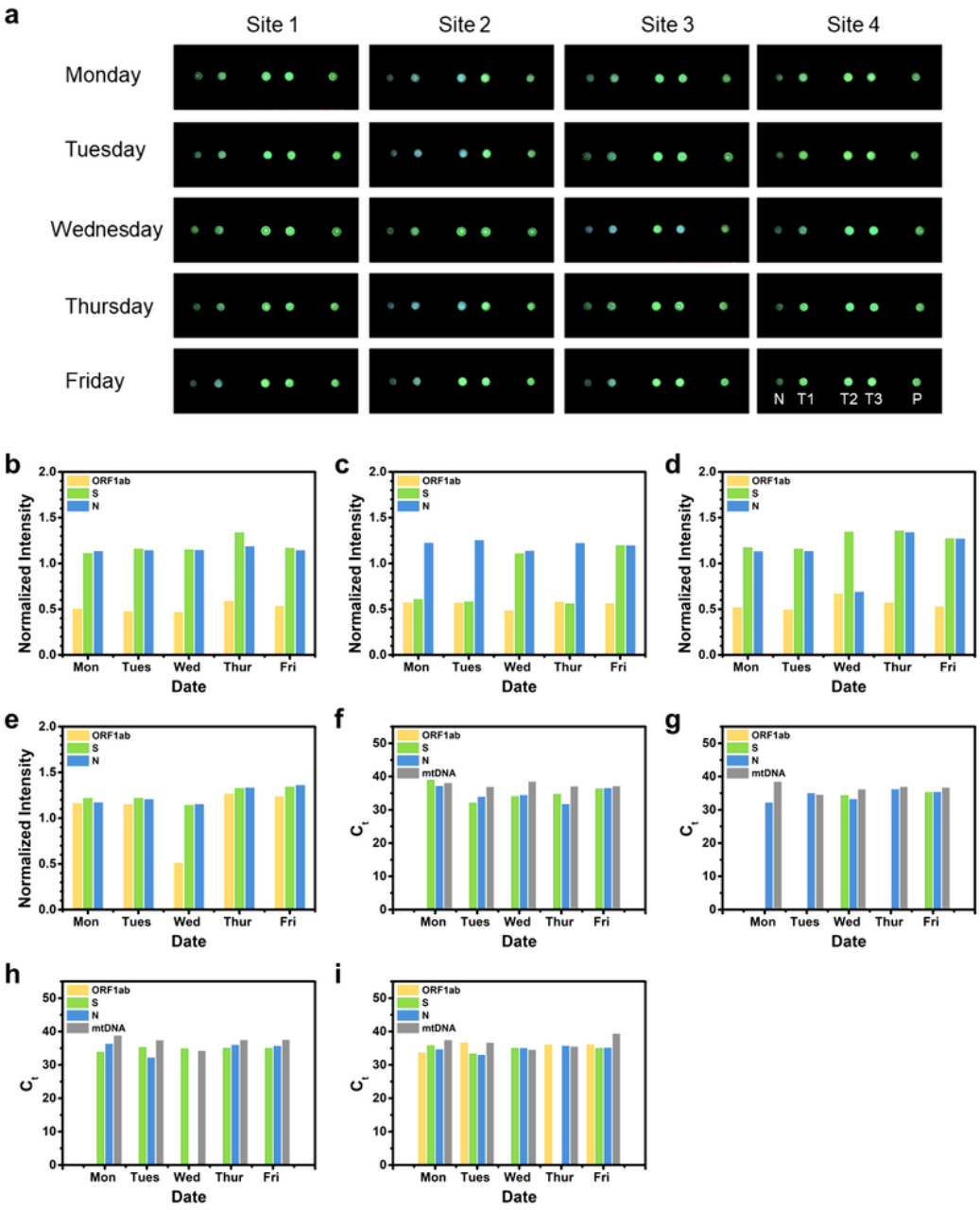

Figure 5

Hotel testing of wastewater samples within the paper-based device. (a) Paper-based RT-LAMP assay for SARS-CoV-2 detection during field testing at four quarantine hotels near Heathrow Airport in London. The

wastewater samples were sampled daily from these four locations near the airport Monday through Friday (5 days). The images of the paper-based RT-LAMP assay were illuminated using a hand-held UV torch and were captured by a mobile phone camera for quantification. N: ddH_2O; T1: ORF1ab; T2: S; T3: N; P: mtDNA. Normalized fluorescence intensity of paper-based RT-LAMP assay for (b) Site 1, (c) Site 2, (d) Site 3, and (e) Site 4. Laboratory-based RT-qPCR detection of SRAS-CoV-2 performed on wastewater samples collected daily from 4 sites near the airport from Monday to Friday, including (f) Site 1, (g) Site 2, (h) Site 3 and (i) Site 4.

Supplementary Files

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