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Development of a field-deployable RT-qPCR workflow for COVID-19 detection

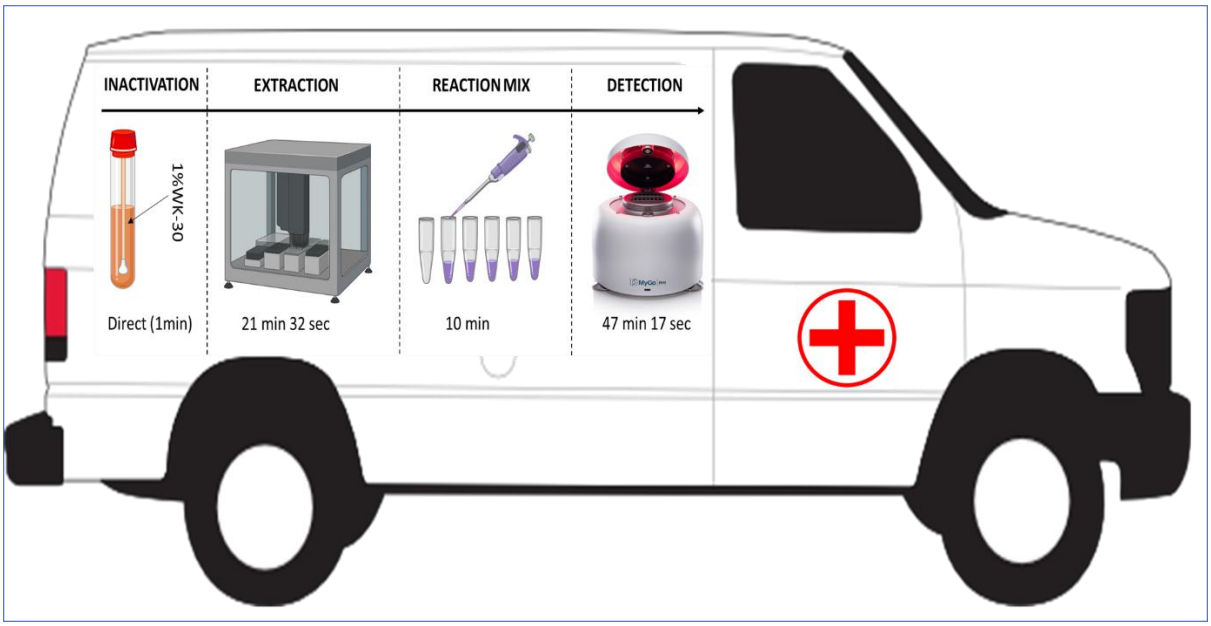
Raphael Nyaruaba[1,2], Bo Zhang[1], Caroline Muema[1,2], Elishiba Muturi[1,2], Greater K. Oyejobi[1,2], Jin Xiong[1], Bei Li[1], Zhengli Shi[1], Caroline Mwaliko[1,2], Junping Yu, Xiaohong Li[1], Hongping Wei[1*]

[1] Key Laboratory of Special Pathogens and Biosafety, Center for Biosafety Mega-Science, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, Hubei, 430071, China

[2] International college, University of Chinese Academy of Sciences, Beijing, 100049, China

[*]**Corresponding author:** Hongping Wei; hpwei@wh.iov.cn; Tel: (+86) 27 51861077; Key Laboratory of Special Pathogens and Biosafety, Center for Biosafety Mega-Science, Wuhan Institute of Virology, Chinese Academy of Sciences, Xiao Hong Shan, #44 Bayi road, 430071, China.

Graphical abstract



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Abstract

Outbreaks of coronavirus disease 2019 (COVID-19) have been recorded in different countries across the globe. The virus is highly contagious, hence early detection, isolation, and quarantine of infected patients will play an important role in containing the viral spread. Diagnosis in a mobile lab can aid to find infected patients in time. Here, we develop a field-deployable diagnostic workflow that can reliably detect COVID-19. Instruments used in this workflow could easily fit in a mobile cabin hospital and also be installed in the community. Different steps from sample inactivation to detection were optimized to find the fastest steps and portable instruments in detection of COVID-19. Each step was compared to that of the normal laboratory diagnosis set-up. From the results, our proposed workflow (80 min) was two times faster compared to that of the normal laboratory workflow (183 min) and a maximum of 32 samples could be detected at each run. Additionally, we showed that using 1% Rewocid WK-30 could inactivate the novel coronavirus directly without affecting the overall detection results. Comparison of our workflow using an in-house assay to that of a commercially acquired assay produced highly reliable results. From the 250 hospital samples tested, there was a high concordance 247/250 (98.8%) between the two assays. The in-house assay sensitivity and specificity were 116/116 (100%) and 131/134 (97.8%) compared to that of the commercial assay. Based on these results, we believe that our workflow is fast, reliable, adaptable and most importantly, field deployable.

Keywords: COVID-19; SARS-CoV-2; field work; community; diagnosis; rapid detection; inactivation; RT-qPCR;

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Introduction

Coronavirus disease 2019 (COVID-19) is caused by the serve acute respiratory syndrome coronavirus 2 (SARS-CoV-2). In December 2019, the first cases of human infection with the Corona Virus Disease 2019 (COVID-19) were identified in Wuhan, China [1–3]. Most of the cases were linked to a local seafood market in Wuhan which is believed to be the source of SARS-CoV-2 virus outbreak [2–5]. Since its identification, scientists have characterized the virus [3,6,7] and reported clinical symptoms associated with COVID-19 [2,3,8,9]. Controlling the virus has been a top priority in areas affected both in China and across the globe.

As of April 8th, 2020, the WHO daily situation report on the novel coronavirus disease recorded a total of 1 353 361 (73 639 new) confirmed cases and 79 235 (6695 new) total deaths resulting from COVID-19 globally [10]. Cases of the epidemic outbreak have also been reported in over 100 countries across the globe [10,11]. With the daily rise in number of deaths, suspected, and confirmed cases across the globe, methods towards rapid diagnosis and detection of the virus disease are of key importance in fighting the pandemic.

From the beginning of the pandemic, different diagnostic approaches have been used in an attempt to tame and understand the spread of infections resulting from SARS-CoV-2 [1,3,8,12,13]. Among these approaches includes development of specific viral nucleic acid assays that use RT-qPCR to diagnose new cases of COVID-19 [1,3,14–16]. Real time qPCR applications in diagnostics however is not a new concept. Since its advent, real time qPCR has become a well-established technique for the diagnosis of various microorganisms owing to its many advantages including rapidity, sensitivity, ease of application, and scalability [17]. Many companies have come up with different qPCR instruments that are lab-based and also field deployable.

In the past corona virus outbreaks [18], RT-qPCR has also been used extensively to help in the identification of different cases and help in the diagnosis pipeline of the virus outbreaks [19–21]. However, in these outbreaks including the ongoing COVID-19, no research has tried to describe an optimized system and workflow suitable for point of care testing, field deployment, and one that can easily fit in a mobile cabin hospital to detect and diagnose the viral disease. Development

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of efficient and quicker methods for detection of viral nucleic acids is said to play an important role in fighting the ongoing corona virus outbreak [12]. Additionally, development of a workflow that can fit in a mobile hospital can guarantee comprehensive healthcare to anybody, in anytime and anywhere [22].

In light of this limitations, we sought to develop a practical workflow that is fast and can be easily adapted in different facilities. The workflow also includes a field-able approach for diagnosis in the community and mobile cabin hospitals set-up. The workflow was compared to the standard workflow using the conventional RT-qPCR diagnosis of the ongoing corona virus outbreak.

Methods

Sample collection

Wuhan Institute of Virology CAS is one of the authorized labs approved by CDC of Wuhan city for detecting COVID-19 in clinical samples. All the samples were handled and deactivated first in biosafety level 2 lab with personal protection equipment for biosafety level 3 lab following the guidelines for detecting nucleic acid of COVID-19 in clinical samples. Research on developing new diagnostic techniques for COVID-19 using clinical samples has been approved by the ethical committee of Wuhan Institute of Virology (2020FCA001).

Human samples in the form of oral swabs were collected from various health facilities across Wuhan and transported to our laboratory for detection. The swab samples were suspended in tubes containing viral transport medium (VTM) prior to transportation. The VTM constituted Hank's balanced salt solution at pH7.4 containing BSA (1%), amphotericin (15 µg/ml), penicillin G (100 units/ ml), and streptomycin (50 µg/ml). Upon receipt at Wuhan Institute of Virology, Zhengdian, the samples were carried in locked containers and moved to the BSL2 laboratory. In the BSL2 lab, the samples were inactivated by heating at 56℃ for 30 min. The samples were then

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left to cool at 4℃ before immediate processing or stored at the same temperature until processing.

Sample processing

To obtain the fastest workflow, we developed a sample matrix of 3 categories for spiking and testing the whole workflow from sample collection to detection. These categories included: 1) Swabs collected from hospitals in VTM tubes; 2) Swabs spiked in RNase free water; and 3) Swabs spiked with already tested positive samples.

The time and data for all the above sample categories were collected in each of the following processing steps:

Sample inactivation

The normal sample inactivation procedure involved heating swab samples in VTM tubes at 56℃ for 30 min and letting potential aerosols to settle for an extra 10 min at 4℃ before extraction. To find a faster way suitable for diagnosis at field conditions, we tried an approach of using the biocidal Rewocid WK 30 (Evonik industries, Shanghai Honenestever Co. Ltd.) here referred to as WK-30 at a concentration of 1% to directly inactivate the virus without heating. The virucidal and bactericidal activity of WK-30 against COVID-19 and different bacterial strains was also tested.

Virucidal activity tests

For virucidal testing, three tests were done independently by mixing 2%WK-30, $1.6x10^6$PFU/ml SARS-CoV-2, and DMEM (*Dulbecco's Modified Eagle Medium*) supplemented with 2%FBS (Fetal Bovine Serum) according to the following ratios: 1) 50µl 2%WK-30 + 50µl 2%FBS DMEM; 2) 50µl SARS-CoV-2 + 50µl 2% FBS DMEM; and 3) 50µl 2%WK-30 + 50µl SARS-CoV-2. The prepared mixtures were left to stand at room temperature for 1 min, and then passed through a sieving column to collect the first 100µl elute. The elutes were then inoculated into the Vero E6 cell and incubated for 48 hours to observe the cytopathic effects, and measure the viral RNA concentration using the RT-qPCR kit.

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Bactericidal activity tests

Five bacterial strains (*Staphylococcus aureus, Escherechia coli, Pseudomonal aeuriginosa, Enterococcus faecalis*, and *Acinetobacter baumannii*) were used in testing the bactericidal effects of WK-30 at two concentrations. The bacterial strains were cultured in LB broth for 24 hours before being transferred to different falcon tubes. The original colony forming units (CFU) of each bacteria was then determined before adding WK-30. After CFU determination, each bacteria was mixed with WK-30 to final concentrations 0.5% and 1% WK-30. After mixing each bacteria with WK-30, the tubes were left to sit for 5, 10, 20, and 30 minutes at room temperature. 10 µl was subsequently collected at each time point and plated onto solid LB media plates. All the plates were then incubated at 37^0 C for 24 hours. Post incubation, the plates were checked to determine the presence of any colonies.

RNA extraction

Four methods of RNA extraction were evaluated to get the fastest method of extraction. The goal was to find a portable extraction method that can easily be used in the field set-up. The four methods included using the Qiagen Viral RNA extraction kit (spin protocol and vacuum protocol), QIAxtractror Automated extraction, and Purifier™ Modesty automated RNA extraction. Each time the extraction methods were used, the manufacturers' recommendations were followed. To determine if WK-30 had an effect in the extraction process, seven hospital samples that had previously tested positive by RT-qPCR were treated with WK-30 to a final concentration of 1% and the RNA extracted. The same samples were also diluted using RNase free water as a control to a final concentration of 1% for testing the normal extraction procedure. The resultant CT values of the two tests were determined using the portable MyGo Pro real time PCR instrument (IT-IS Life Science Ltd., Mahon, Cork, Ireland).

Sample detection

Different approaches were also considered for sample detection. These include:

RT-qPCR quantification instruments

To find a suitable qPCR instrument that is field deployable and suitable for POC testing, we used a portable MyGo Pro Real-time PCR instrument readily available in our laboratory to optimize

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our workflow. The machine detection time was compared to that of the routinely used CFX96 Touch™ Real-time PCR (CFX96, Bio-Rad Laboratories, Hercules, CA, USA) instrument suitable for laboratory bench top diagnosis.

RT-qPCR assay composition and reagent preparation

Two assays were used for time comparison and workflow tests. The two assays comprised of a commercially acquired kit for RT-qPCR and an in-house assay developed using locally available reagents to perform RT-qPCR.

The commercially acquired kit was labelled New Coronavirus 2019-nCoV Nucleic Acid Detection Kit (Fluorescence PCR method) produced by the Zhongshan Daan Gene Company, Guanzhou. The kit is a one-step RT-qPCR kit designed to target the open reading frame 1ab (ORF1ab) and nucleocapsid protein (N) genes of the novel coronavirus sequence. The N gene was labelled with a FAM reporter dye while the ORF1ab gene was labelled with a VIC reported dye. The kit also had an endogenous internal control dye labelled Cy5. The 25 µl reaction mixture consisted of 20 µl of freshly prepared mix and 5 µl RNA template. The one-step RT-qPCR protocol was run using the Bio-Rad's CFX 96 instrument under the following conditions: 50 ℃ for 15 min, 95 ℃ for 15 min, followed by 45 cycles of 95 ℃ for 15 sec and reading at 55 ℃ for 45 sec respectively.

The in-house assay was a one-step RT-qPCR assay composed of two sets of primers and probes. A set of primers was designed to target the receptor binding domain (RBD) of the novel corona virus sequence while the other set was designed to detect an endogenous internal control (IC). The primers and probe targeting the RBD sequence include: forward primer CTCAAGTGTCTGTGGATCACG; reverse primer CCTGTGCCTGTTAAACCATTG; and probe 5`-FAM-ACAGCATCAGTAGTGTCAGCAATGTCTC-BHQ1-3`. The IC primers and probe sequence: forward primer AGATTTGGACCTGCGAGCG; reverse primer GAGCGGCTGTCTCCACAAGT; and probe 5`-FAM-TTCTGACCTGAAGGCTCTGCGCG-BHQ1-3`. Before addition, all the primer and probe concentrations were adjusted to 10 µM. The composition of the in-house assay included O.8 µl of each primer, 1 µl of each probe, 2 µl 5× PrimeScript RT Master, 1.6 µl dNTP, 0.5 µl Taq polymerase, 3.7 µl RNase free water, and 5 µl RNA template to a final volume of 20 µl. All the in-house one-step RT-qPCR reaction mixtures were then amplified using the MyGo Pro Real time

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PCR instrument under the following conditions: 50 ℃ for 10 min, 95 ℃ for 10 sec, followed by 40 cycles of 95 ℃ for 5 sec and reading at 60 ℃ for 30 sec respectively.

Assay specificity and performance

For specificity testing of the in-house assay, an *in silico* test using the basic local alignment search tool (BLAST) available online from NCBI was used. To test whether there will be a remarkable difference between the in-house assay and the commercial kit, 250 hospital samples were extracted and used for detection. The commercially available kit was used as a reference kit for validating the workflow of the in-house assay. All the commercial kit tests were done using the Bio-Rad's CFX-96 real-time PCR instrument while all the in-house assay tests were done using the portable MyGo Pro real-time PCR instrument.

Results

Sample inactivation

Two approaches were used to determine the fastest sample inactivation procedure that may be suitable for field applications and POC testing. The normal routine sample inactivation procedure which involved heating the swab samples suspended in VTM tubes, took up to 40 minutes to inactivate the sample. The inactivation process also needed a heating source and cooling source for inactivation. Hence, this was not readily suitable for field deployment. Therefore a direct approach using the biocidal surfactant WK-30 was explored. The surfactants ability to kill different bacteria and SARS-CoV-2 was established.

The virucidal effect of 1% WK-30 against SARS-CoV-2 was tested *in vitro* using Vero E6 cells as shown in **Figure 1.** From the results, it was clear that 1% WK-30 had no effect on Vero E6 cells even after 48 hours of incubation as shown in **Figure 1 A**. However, incubating the cells with $1.6x10^6$ PFU/ml SARS-CoV-2 proved to be lethal. All the cells were dead after 48 hours incubation with clear cytopathic effects (CPE) as shown in **Figure 1 B**. Most of the cells were alive with no CPE after 48 hours incubation with both 1% WK-30 and $1.6x10^6$PFU/ml SARS-CoV-2 as shown in **Figure 1 C**. Additionally, the Ct values were quantified at time 0 hours and 48 hours of incubation.

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The Ct greatly dropped from Ct 19.51 at time 0 hours to Ct 34.46 at time 48 hours. From these results, 1% WK-30 showed some level of protection to the cells. Also interaction of 1% WK-30 with SARS-CoV-2 for 1 min at room temperature can kill all of the SARS-CoV-2.

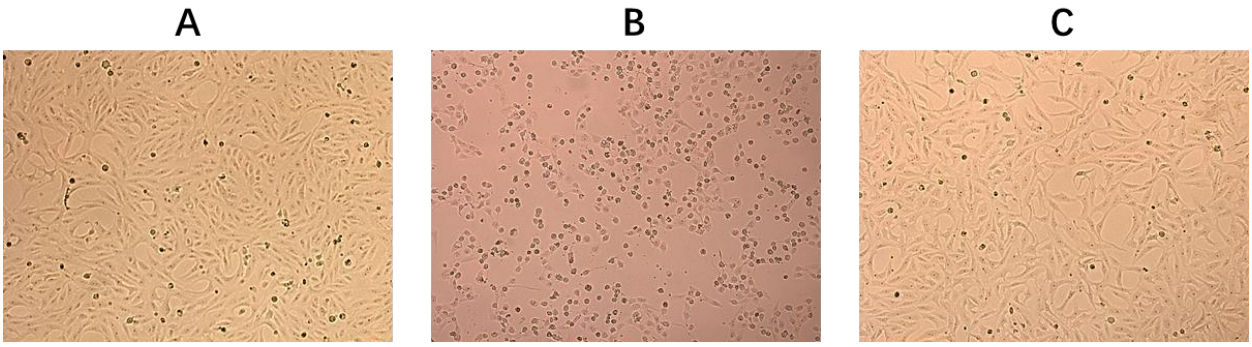

Figure 1: Virucidal effect of 1% WK-30 against the novel coronavirus. A) Most of the cells are alive and with no CPE after 48 hours incubation with 50µl 2%WK-30 + 50µl 2%FBS DMEM. B) All the vero E6 cells are dead with CPE after 48 hours incubation with 50µl 2019-nCoV + 50µl 2% FBS DMEM. C) Most cells are alive with no CPE after 48 hours incubation with 50µl 2%WK-30 + 50µl 2019-nCoV.

For the bactericidal effects, five bacteria were used. From the results (**Table 1**), all the colonies reduced with increased exposure time to WK-30. *E. coli*, *E. faecalis*, and *A. bauminii* were completely inhibited by 0.5%WK-30 at all time points. However, *S. aureus* and *P. aeruginosa* showed some level of resistance to 0.5%WK-30. *S. aureus* was the most resistant bacteria because even after 30 min of exposure, some colonies still grew post incubation. Of note, 1%WK-30 however was able to kill all the bacteria as no colonies were observed post incubation. 1%WK-30 had a maximum bactericidal effect to all the bacterial strains.

From this results, 1% WK-30 was both bactericidal and virucidal hence suitable for use in the field as it requires no extra sample treatments and reduces the time needed for inactivation and sample transportation.

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CFU before addition of WK30	*S. aureus*		*E.coli*		*P. aeruginosa*		*E. faecalis*		*A. baumannii*	
	$3.3*10^8$		$5.1*10^7$		$7.7*10^6$		$4.3*10^8$		$3.7*10^8$	
Conc of WK30	**0.5%**	**1%**	**0.5%**	**1%**	**0.5%**	**1%**	**0.5%**	**1%**	**0.5%**	**1%**
CFU after 5 mins	TNTC	0	0	0	$8*10^2$	0	0	0	0	0
CFU after 10mins	TNTC	0	0	0	$1*10^2$	0	0	0	0	0
CFU after 20mins	$3.31*10^4$	0	0	0	0	0	0	0	0	0
CFU after 30mins	$6.9*10^3$	0	0	0	0	0	0	0	0	0

TNTC- too numerous to count

Table 1: Bactericidal effect on different bacteria using 0.5% and 1% WK-30.

RNA extraction

Different methods were compared to find the fastest, portable and easy to use RNA extraction mechanism in a field setup. Factors including time, number of sample handling steps, and any additional instrument needed were also determined as shown in **Table 2**.

Method/ Instrument	Extraction Kit	Automated	Portable	Manual steps	Centrifugation steps	Samples	Time (Min)
Purifier™ Modesty	Genfine	Yes	Yes	None	None	32	21
QIAxtractor	QIAamp® 96 QIAcube® HT Kit (5)	Yes	No	None	None	96	120
Spin protocol	QIAamp Viral RNA Mini Kit	No	Yes*	Multiple	All	Flexible	~70
Vacuum protocol	QIAamp Viral RNA Mini Kit	Partially	Yes*	Multiple	2	24	~ 40

* May be portable provided there is a source of centrifugation and biosafety cabinet.

Table 2: Comparison of four extraction methods. Purifier™ Modesty automated instrument used the least time to extract samples with minimal sample handling steps.

The comparison revealed that using the Purifier™ Modesty instrument was faster and needed less sample handling steps compared to other methods. The instrument was easily portable and

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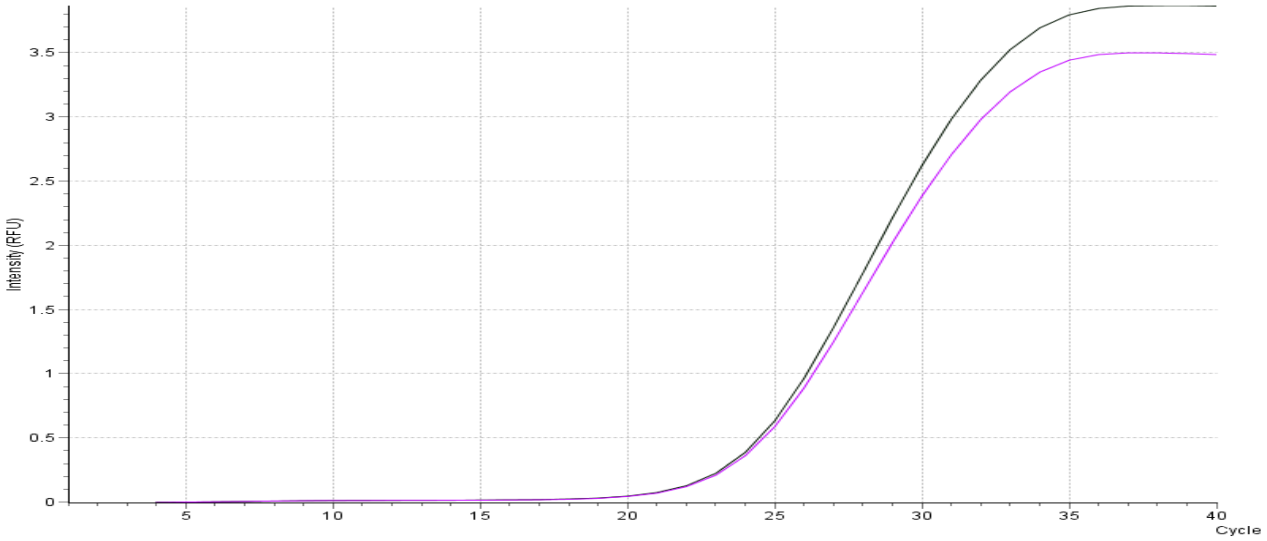
fitted with a UV lamp that can help in decontamination and ensuring a clean environment. Due to these advantages, the instrument was found suitable for field deployment.

Since 1%WK-30 was found to be virucidal, we tested if the solution would have an effect in the extraction process when using the Purifier™ Modesty instrument. The result of addition of 1% WK-30 to the virus was tested in comparison of using 1%RNase free water in the same sample to replace WK-30. The results as shown in **Table 3 and Figure 2** proved that 1%WK-30 had no remarkable effect in the extraction process and the results were highly comparable.



Sample	Ct value of samples spiked with	
	1%WK-30	1%RNase free water
20200126-06	32.604/32.606	32.273/32.525
20200126-07	23.253/23.198	23.142/23.146
20200126-09	34.444/35.174	34.392/35.655
20200126-17	31.415/31.105	31.967/31.276
20200126-23	28.914/28.764	29.02/28.837
20200126-25	26.719/26.593	28.186/28.277
20200126-26	29.707/29.928	30.602/30.807

Figure 2 and Table 3: Figure 2 is a representation of the RT-qPCR results after extraction with 1%WK-30 (black curve) and 1%RNase free water (purple). Table 3 shows the results of seven

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already tested positive samples. In both Table 3 and Figure 2, the results show that 1%WK-30 has no effect in the extraction process and overall result.

Detection time

To find the shortest time that could detect samples faster and reliably, we modified our protocol to run for a total time of 47min 17sec using the MyGo Pro instrument. This time was 64 min 2sec shorter compared to that of the commercially acquired New Coronavirus 2019-nCoV Nucleic Acid Detection Kit which run for 1hour 51min 37sec according to the manufactures' procedure.

Assay sensitivity and specificity

An *in silico* probe and primer test using the basic local alignment search tool (BLAST) available online from NCBI resulted to 100% specificity of the in-house primers and probes in detection of the novel coronavirus. The performance of the whole workflow including reagents was tested using 250 patient samples. The result of this test is summarized in **Table 4.**

Commercial lab assay	**Field-deployable assay**	
	Positive	**Negative**
Positive	116	0
Negative	3	131
Sensitivity	100%	
Specificity	97.8%	

Table 4: Agreement of the commercial lab assay and the field-deployable assay for detection of swabs from suspected patients with COVID-19 infection.

From the results (Table 4), the novel coronavirus was detected in 116/250 (46.4%) hospital samples using the commercial kit. The in-house assay was a little more sensitive than the commercial assay as 119/250 (47.6%) hospital samples tested positive for the novel coronavirus using the in-house assay. Of note, the concordance between the two assays was high 247/250 (98.8%). However, three samples that tested negative by the commercial assay tested positive by the in-house assay resulting to a specificity of 97.8%. All the samples that tested positive by the commercial assay also tested positive by the in-house assay 116/166 (100% sensitivity)

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indicating that the in-house assay was greatly comparable to the commercial assay with improved sensitivity.

Discussion

Since the discovery of the first case of COVID-19 in Wuhan, China [1–3], scientists have been working hard to diagnose the disease. Efforts geared towards diagnosis of COVID-19 have been patient centered and applied in hospital set-ups. However, with the rising numbers of cases across the globe [11,23,24], more efforts should be explored especially in community diagnostics. There may be a number of cases being missed from patients in the community hence field work tests may prove beneficial in treatment and diagnosis of COVID-19. A field based diagnostic approach has not yet been explored. In this article, we used locally available instruments and reagents to develop a rapid RT-qPCR diagnostic workflow that is not only suitable for POC, but also field deployable.

In the field set up, a rapid direct sample inactivation method that needs no additional instruments could be of great importance. To develop such a method, we explored the biocidal effects of Rewocid WK 30 [25]. We tested if the surfactant had a potential of killing both bacteria and viruses. In both cases, 1%WK-30 was shown to be virucidal to SARS-CoV-2 and also bactericidal to different bacteria *in vitro*. The virucidal effects of 1%WK-30 also had no effect in the extraction process. So far, no literature has cited or tried to explore the biocidal capabilities of Rewocid WK 30. The ability of 1%WK-30 to inactivate and kill the novel coronavirus at room temperature within a minute of exposure makes it a suitable reagent for direct sample inactivation in the field set-up.

Once the sample has been inactivated, the sample should proceed directly to extraction of the viral nucleic acid necessary for detection. A fast, portable automated extractor with minimal sample handling steps will be suitable for field extraction experiments. Automation with minimal sample handling steps ensures safety to the worker and also ensures sample integrity is kept intact throughout extraction in the field where there are minimal or no specialized equipment e.g. biosafety cabinets. A portable instrument on the other hand meant that it could fit well in a

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mobile hospital. Of all the available extraction methods and instruments tested in this workflow, the Purifier™ Modesty instrument was the fastest and most portable. The instrument was also fitted with a UV light source to ensure the cleanliness of the extraction environment pre and post extraction. Up to 32 samples could be extracted in a minimal time of about 21 minutes. This was highly scalable as 32 patients could be served in a single run of extraction.

To make the workflow complete, we coupled the workflow with a fast detection protocol using a portable MyGo Pro real time PCR instrument for detection. The instrument was highly compatible with the Purifier™ Modesty nucleic acid extractor as it could also process a maximum of 32 samples. The fast RT-qPCR protocol ensured samples were detected within 47min 17sec. This detection time was faster and results highly comparable with that of the commercial assay. The MyGo Pro real time PCR instrument could be run with a USB drive. This meant that multiple instrument could be used and run with only a single computer using multiple USB programmed drives. The multiple options for running the PCR instrument gave room for scaling.

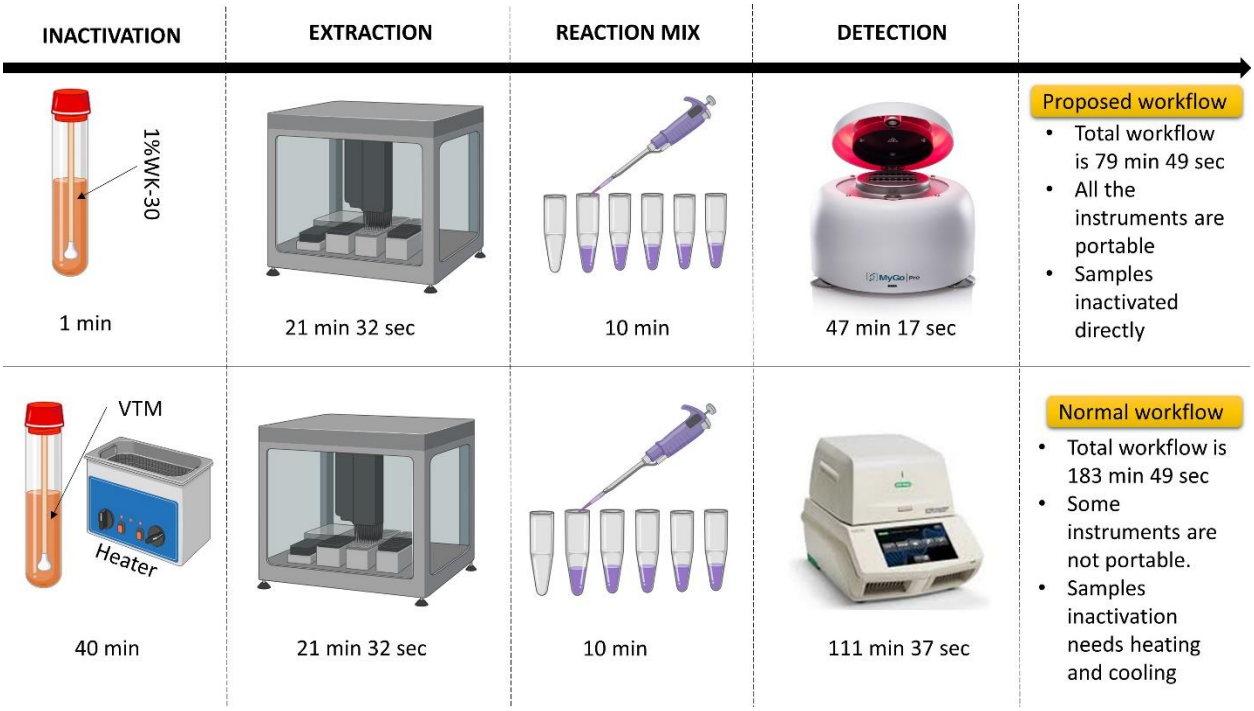

Figure 3: Schematic comparison of the proposed workflow compared to the normal lab-based detection workflow. The proposed workflow is faster than the normal lab-based workflow. All the instruments in the proposed workflow are portable.

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From **Figure 3**, all the instrument used in optimization of the proposed workflow were highly portable. This meant that the workflow instruments could easily be fitted and adapted in a mobile hospital before deployment to the community to detect and diagnose COVID-19. The complete workflow from sample inactivation to detection was approximated to be no more than 80 minutes using the field deployable workflow (**Figure 3**). This workflow was two times faster compared to that of the normal workflow in the laboratory set up (183 min 9 sec). Additionally, after the first detection of approximately 80 minutes, the proceeding samples can be extracted and detected within an hour. As the first samples are running a new batch of samples may be loaded into the extractor which will run for 21 min, the extra 26 minutes before the RT-qPCR results are read can be used for reaction mix preparation and programming. Once the first batch finishes, the newly extracted samples can be detected immediately.

This workflow is a first in exploring the potential of community diagnosis using a field deployable nucleic acid detection system. All the instruments highlighted to be used in the field set-up are highly portable and can fit well in a mobile hospital. Additionally, the workflow is flexible and can be modified depending on the available resources in different laboratories and countries. We plan to actualize this workflow in our subsequent tests using a mobile hospital to help in the diagnosis of COVID-19. Actualization of this workflow ensures that patients are treated anywhere and at any time. This will also help in reducing the large number of patients that visit hospitals. Patient treatment at community level decreases their risks of getting infected when visiting the hospitals. Different researchers and laboratories can use our workflow and modify it if needed to best fit their laboratory.

Conclusion

Portable instruments that can be used in extraction and detection of nucleic acids already exist. We used locally available instruments and reagents to develop a field-deployable workflow that can also be used at POC. We found that using Rewocid WK 30 as a biocidal agent could help in inactivation of SARS-CoV-2 samples directly without the need of any extra equipment. This workflow was two times faster than that of the normal workflow in the laboratory set-up. We believe that the workflow described is easily adaptable and flexible. This workflow will help in

the fight against the current SARS-CoV-2 pandemic and other future outbreaks. Lastly, the workflow may be useful in routine sample collection and detection in future field-work surveillance studies.

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Conflicts of Interest: The authors declare no conflict of interest.

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