

Direct lysis RT-qPCR of SARS-CoV-2 in cell culture supernatant allows for fast and accurate quantification of virus, opening a vast array of applications

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ABSTRACT

An enormous global effort is being made to study SARS-CoV-2 and develop safe and effective treatments. Studying the entire virus replication cycle of SARS-CoV-2 is essential to identify host factors and treatments to combat the infection. However, quantification of released virus often requires lengthy procedures, such as endpoint dilution assays or reinfection with engineered reporter viruses. Quantification of viral RNA in cell supernatant is faster and can be performed on clinical isolates. However, viral RNA purification is expensive in time and resources and often unsuitable for high-throughput screening. Here, we show a direct lysis RT-qPCR method allowing sensitive, accurate, fast, and cheap quantification of SARS-CoV-2 in culture supernatant. During lysis, the virus is completely inactivated, allowing further processing in low containment areas. This protocol facilitates a wide array of high- and low-throughput applications from basic quantification to studying the biology of SARS-CoV-2 and to identify novel antiviral treatments *in vitro*.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19), which emerged towards the end of 2019 (Wu et al., 2020). From an original outbreak in China, the virus spread rapidly across the globe, leading The World Health Organization to assign the virus pandemic status in March 2020. Since then, millions of confirmed cases and deaths have been associated with SARS-CoV-2 infection.

SARS-CoV-2 is an enveloped, positive-sense RNA virus and belonging to the family *Coronaviridae* in the order *Nidovirales*. Coronaviruses (CoVs) are capable of infecting a wide variety of mammalian and avian species. In most cases, they cause respiratory and/or intestinal tract disease. Human coronaviruses (hCoVs) are known as major causes of the common cold (e.g. HCoV-229E and HCoV-OC43). However, the emergence of new hCoVs of zoonotic origin has shown the potential of CoVs to cause life-threatening disease in humans, as was demonstrated during the 2002/2003 SARS-CoV-1 epidemics, the ongoing MERS-CoV epidemics in the Middle East, and now the SARS-CoV-2 pandemic (Peiris et al., 2003; Zaki et al., 2012).

The global vaccination effort has eased the burden of COVID-19 slightly, but there remains an urgent need for effective anti-viral treatments, especially for early administration, outpatient treatments to prevent progression to severe disease, particularly in high-risk patients. Early efforts to identify interventions inhibiting SARS-CoV-2 replication relied on the repurposing of existing, approved drugs with known toxicity profiles rather than *de novo* drug development. Whilst hundreds of drugs were trialed in hundreds of thousands of patients, only a panel of two drugs were given a grade A by the CORONA Project database in treatment efficacy and/or research prioritization for outpatient treatments: Bamlanivimab+Etesevimab and Sotrovimab (A for both) and Budesonide (A for research prioritization, B/C in treatment efficacy), and Fluvoxamine (A for research prioritization) (<https://cdcn.org/corona/>, accessed 30/11/2021). It is therefore essential to continue the effort to find new and repurposed treatments against SARS-CoV-2 infection. Examining the whole replication cycle, including virus release, will reveal more candidates for further investigation and narrow the search to drugs that truly reduce the viral load.

In both diagnostic RT-qPCR and *in vitro* studies of SARS-CoV-2, the requirement to extract and purify viral RNA (vRNA) prior to measuring virus RNA copy numbers is expensive in both time and resources. Methods for lysis and direct RT-qPCR of viral samples without RNA purification have previously been developed for the study of influenza (cell lysate, (Shatzkes et al., 2014)), Dengue virus (cell supernatant (Suzuki et al., 2018)), Zika virus (patient samples, (Li et al., 2019)), norovirus and hepatitis A Virus (foods, (Rajjuddin et al., 2020)). In some of these, the use of expensive, commercially available lysis buffers limits applicability for high-throughput, and none of the publications analyzed the impact of lysis buffer on efficiency and sensitivity of the assays. For SARS-CoV-2, several groups have developed direct RT-qPCR methods for detection aimed at patient swabs. The most commonly used method is direct use of swabs following a heating step, 30min at 65°C or increasingly shorter periods up to 95°C. With or without addition of commercial buffers or detergents, a Ct difference between 4-7 cycles were observed compared with extracted vRNA (Alcoba-Florez et al., 2020; Bruce et al., 2020; Fomsgaard and Rosenstierne, 2020; Genoud et al., 2021; Grant et al., 2020; Hasan et al., 2020; Nique et al., 2021; Pearson et al., 2021; Smyrlaki et al., 2020). Other methods include the addition of proteinase K to patient swab samples, showing 4-6 cycle differences, but no proof of virus inactivation was shown for these samples (Mallmann et al., 2020; Srivatsan et al., 2021). Commercial kits or homemade detergent-based kits showed good correlation with positive clinical samples but virus inactivation and loss of Ct were not determined (Castellanos-Gonzalez et al., 2021; Ladha et al., 2020; Merindol et al., 2020). The reduction in sensitivity, lack of inactivation proof, or the reliance on expensive proprietary lysis buffers makes many of these methods unsuitable for quantification in *in vitro*-amplified viral culture supernatants. Here, we show a method for direct lysis and RT-qPCR of vRNA in culture supernatant using a cheap, non-commercial IGEPAL CA-630 (IGEPAL-630)-based lysis buffer which completely inactivates SARS-CoV-2 (>1E6 TCID₅₀/ml reduction). The assay shows high sensitivity, detecting <0.0043 TCID₅₀ per reaction in lysate and to <1.89 copy per reaction using RNA template-spiked mock lysate. The method described here can be used to accurately, rapidly and cost-effectively quantify SARS-CoV-2 production in cell culture supernatant, allowing for faster workflows, saving time and resources on routine virological applications as well as high-throughput screening.

RESULTS

SARS-CoV-2 primer optimization using a 1-Step-RT-qPCR fluorescent dye system shows highest efficiency and sensitivity with the CDC N3 primer.

1-Step-RT-qPCR using fluorescent dye detection is by far the most cost effective method of RT-qPCR, therefore, we focused on optimizing this for use with *in vitro* applications requiring quantification of SARS-CoV-2 production. Primer pairs N1, N2, and N3 by the US Centers for Disease Control and Prevention (CDC) (Lu et al., 2020) and the German Center for Infection Research (DZIF) against RdRp and N (Corman et al., 2020), designed and widely used for the detection of SARS-CoV-2, were selected for optimization in a 1-Step-RT-qPCR reaction using the Promega GoTaq system incorporating the dsDNA-binding dye BYRT green on a LightCycler480.

Increasing concentrations of primer (symmetric and asymmetric concentrations) were tested following the manufacturer's standard protocol, using target-specific, *in vitro* transcribed RNA templates of 600-1600bp length in 10µl and 20µl final reaction volumes. An increase of the reaction volume to the recommended 20µl showed no difference in efficacy or sensitivity for any of the primers (data not shown), therefore data shown here illustrates the more cost-effective 10µl reaction volume.

All primer pairs tested showed clear improvement in sensitivity from 50 to 250nM of equal primer concentration (Figure 1A and C). Efficiencies, calculated by the LinRegPCR program (Version 11.0, (Ruijter et al., 2009)), showed a similar increase (Figure 1C). Detected fluorescence was highest for CDC N3, DZIF N, and RdRp (Figure 1A), which can be partly explained by the longer product length of DZIF N (128bp) and DZIF RdRp (100bp), but also the higher reaction efficiency, which can be observed by comparing the difference in CDC N primers, all amplifying a roughly 70bp product (Figure 1C). Using standard conditions with 60°C annealing temperature, CDC N1, DZIF N and RdRp consistently showed the formation of primer dimer products (Figure 1B). Increasing the concentration of N2 to the recommended concentration of 1,000nM improved its efficiency, but resulted in intermittent primer dimer formation (data not shown).

Analyzing the bands generated in the PCR reaction, we found that DZIF N formed larger products than the expected 128bp (Figure 1D). Sanger sequencing was only successful on some of the products but revealed circularization or multiplication of a section of the product. Increasing the annealing temperature did not eliminate the occurrence of these products.

Despite the use of significantly increased primer concentrations for the DZIF N primer, efficiencies did not improve beyond 78.24%. DZIF RdRp showed an efficiency of 89.61% (Figure 1E). However, by far the best performing was primer pair CDC N3 at a symmetrical concentration of 350nM showing an efficiency of 93.14% and a sensitivity of <1.89 template copies/ reaction (Figure 1E). Paired with the production of primer dimer by CDC N1 and N2, and DZIF RdRp, and the multiple products of DZIF N, CDC N3 at a symmetrical concentration of 350nM forward/reverse was selected for further development of the method.

IGEPAL-630-based buffers show highest efficiency and sensitivity, shortly followed by Triton X-100.

As a first step, heat lysis, as described for patient samples, was tested towards release of vRNA from the capsid in virus culture supernatant, hereafter referred to as virus production medium (VPM). However, vRNA release from VPM by heating for 5min at 95°C was found to be limited. The difference in Ct values between vRNA extracted using a column RNA purification kit and heat lysis of an equivalent corresponding volume of VPM was found to be >10 cycles, corresponding to a roughly 1,000x loss in sensitivity (data not shown). Combined with the requirement for a heat block or, ideally, a PCR machine to ensure correct core temperature during heat inactivation and concerns over RNA stability, heat inactivation was abandoned as a broadly applicable method and focus shifted to a detergent lysis-based method.

Initial optimization experiments were performed using VPM following heat inactivation, at 70°C for 10min using a PCR machine to ensure good heat transfer and correct core temperature, to allow processing outside CL3. This virus inactivation method had previously been confirmed by serial dilution

and inoculation of cells with heat-inactivated virus ($>6\log_{10}$ TCID₅₀/ml reduction). At later stages, once virus inactivation by lysis buffer was confirmed, we validated that there was no difference in Ct values, sensitivity, or efficiency between vRNA extracted from VPM with and without 70°C heat-inactivation (data not shown).

All tested lysis buffers were based on a 150mM NaCl, 10mM Tris-HCl, pH 7.5 solution supplemented with different lysis detergents for VPM to be lysed at a 1:1 ratio. The salt concentration in the lysis buffer was based on (Shatzkes et al., 2014), who found a 150mM NaCl concentration to be most sensitive in the RT-qPCR reaction. VPM, which in most cases is DMEM or RPMI, contains 108-118mM Cl⁻ and 138-155mM Na⁺, thus in a 1:1 dilution with lysis buffer, NaCl concentration will remain just slightly under 150mM.

Three different detergents were tested to assess their efficacy in releasing vRNA from VPM for RT-qPCR: 1 or 10% Triton X-100, 0.25% IGEPAL-630, or 5% Tween-20 in a buffer supplemented with 10U/ml RNasin Plus RNA inhibitor. These initial concentrations were chosen either based on available SARS-CoV-2 inactivation data or previous reports in lysis protocols. VPM lysis was performed for 20min at room temperature. It was also assessed whether proteinase K treatment could improve vRNA release. Therefore, proteinase K was added at 0.1AU/ml to each detergent lysis buffer, supplemented with 0.83mM final concentration EDTA to prevent heat damage to RNA during heat inactivation, mixed 1:1 with VPM or vRNA in nuclease-free water (NF-H₂O), and incubated for 30min at 56°C prior to heat inactivation for 10min at 95°C. Proteinase K is not inactivated by EDTA and has been shown to be active in high detergent buffers.

1% Triton X-100, 5% Tween-20, and 0.25% IGEPAL-630 detergent lysis buffers showed similar sensitivities (Cts 14.75-15.01) compared to purified equivalent amounts of vRNA (Ct 13.53). The qPCR reaction was clearly impaired by 10% Triton X-100, and 5% Tween-20 buffers, which consistently produced a lower fluorescence. When the detergent lysis buffers were tested in combination with heating with proteinase K for vRNA or in VPM lysis alone, a marked increase by 1.22-1.37Cts was observed. This increase was even higher for 10% Triton X-100, where proteinase K digest lead to a loss of 5.86Ct. Efficiencies as calculated by LinRegPCR were above 78% using 0.25% IGEPAL-630, 1%

Triton X-100, or 5% Tween-20. 10% Triton X-100, however, shows markedly lower efficiencies (59.2-62.89%) in VPM. All detergents used are likely to work at high efficiencies if used at lower detergent concentrations upon further optimization. We decided to continue optimization of the IGEPAL-630 buffer based on comparable sensitivity at lower concentration to the other detergents. (Figure 2A)

Increasing IGEPAL-630 concentration to 2.5% improves RT-qPCR efficacy and inactivates SARS-CoV-2 whilst not affecting sensitivity

To improve virus lysis, buffers containing increasing concentrations of 0.25 %, 0.5 %, 2.5 %, and 4% IGEPAL-630 were tested containing 10U/ml RNasin Plus RNase inhibitor. VPM was lysed for 20min at room temperature before adding 1µl of the 1:1 VPM/lysis buffer to a 10µl total reaction volume for RT-qPCR analysis. Efficiency decreased slightly with higher IGEPAL-630 concentrations (83.16% at 0.25% to 77.35% at 4% IGEPAL-630). Ct difference compared to an equivalent amount of vRNA was found to be 2.85 cycles in these experiments so further optimizations were necessary to improve sensitivity. (Figure 2B)

To test inactivation of SARS-CoV-2 by the lysis, samples were incubated with 0.25 and 2.5% IGEPAL-630 lysis buffer for 20min. A serial dilution of the lysate or a purified version where the lysis buffer had been exchanged in a 100kDa protein concentrator with PBS, as well as the VPM without lysis were inoculated onto a confluent layer of VeroE6 cells. Cell supernatant was replaced 1.5 hours post inoculation (hpi) and supernatant harvested 72hpi. Produced virus in the supernatant was quantified by RT-qPCR. 0.25% IGEPAL-630 only reduced SARS-CoV-2 infectivity by >4log₁₀ TCID₅₀/ml, however, 2.5% IGEPAL-630 (at a final concentration of 1.25% in the lysate), showed complete inactivation with a reduction of >6log₁₀ TCID₅₀/ml (Table 1).

To test the effect of IGEPAL-630 on the RT-qPCR reaction, a small amount of template RNA (189 copies/reaction) was tested in an RT-qPCR reaction diluted 1:1 in 2.5% IGEPAL-630 buffer or in NF-H₂O, and added at 1µl/reaction. No significant decrease in sensitivity could be observed, however,

efficiency was decreased both with lower fluorescence and LinRegPCR-calculated efficiency (Figure 2C).

RNases and RT-qPCR inhibitors in VPM can contribute to the reduced sensitivity of the direct lysis RT-qPCR reaction

To elucidate why sensitivities in direct lysis VPM RT-qPCR were lower than in extracted vRNA, despite IGEPAL-630 on its own not affecting sensitivity, a series of experiments were performed. A 10-fold dilutions series of RNA template were quantified, diluted in either NF-H₂O or mock lysate (2.5% IGEPAL-630 mixed 1:1 with fresh cell culture media to replicate a lysate). Cts were increased by an average of 8.75 cycles across all concentrations in samples containing media. Only a slight loss of efficiency could be observed in the dilution curve. (Figure 2D).

A small amount of RNA template was diluted 1:10 in either NF-H₂O, 2.5% IGEPAL-630 lysis buffer, fresh cell culture medium or a 1:1 mixture of fresh media and lysis buffer to mimic a lysate, with or without increasing concentrations of RNase inhibitor, incubated for 20 minutes and tested in an RT-qPCR reaction at 1µl/reaction (yielding 18.9 copies/reaction). Incubation in 2.5% IGEPAL-630 lysis buffer or fresh media alone led to an increase in Ct of 3.69 or 2.54 cycles respectively compared to NF-H₂O, while incubation in the mixture of both resulted in an increase of 6.29 cycles. This decrease in sensitivity was completely rescued by the addition of as low as 10U/ml RNasin to the media/2.5% IGEPAL-630 lysis buffer indicating the presence of RNases in both the media and the lysis buffer. (Figure 2E)

When the effects of RNase inhibitors on VPM lysate (using 2.5% IGEPAL-630 lysis buffer) were examined, they were not as pronounced as the effects on the mock lysate using fresh cell culture medium. Incubation for 20 minutes with a high concentration of RNase inhibitors still leads to an increase in Ct of 2.63-2.71 cycles, indicating the presence of RT-qPCR inhibitors in the “spent” but not “fresh” media. (Figure 2E)

Neither Betaine addition nor increased primer concentrations improve direct lysis RT-qPCR sensitivity.

To try to improve direct lysis RT-qPCR efficiency in the face of suspected inhibitors introduced during cell and virus culture, we tested the addition of betaine, known to improve PCR amplification by relaxing secondary structures, and its effect on the direct lysis RT-qPCR reaction. 2.5% IGEPAL-630 lysis buffer containing 10U/ml RNasin Plus was tested in combination with increasing amounts of betaine in the RT-qPCR reaction. 1M Betaine decreased the efficiency of the RT-qPCR significantly, whilst lower concentrations (0.5M and 0.1M) showed no decrease in efficiency but a slight decrease in signal. Overall, betaine addition showed no improvement in sensitivity. (Figure 2F)

To assess whether the primer concentrations of CDC N3 used were still appropriate for VPM lysate, and whether further improvements in sensitivity and or efficacy could be made, we tested increasing concentrations of primers ranging from 350 to 600nM in direct lysis RT-qPCR with 2% of the total volume comprising VPM/2.5% IGEPAL-630 lysate with 10U/ml RNase inhibitor. No asymmetric primer concentrations were tested. Increasing concentrations of primer did not significantly increase sensitivity and only marginally increased efficiency when analyzed using LinRegPCR (Figure 2G). A serial dilution of VPM lysate at 2% lysate per reaction with 350nM symmetrical forward and reverse CDC N3 primer concentrations showed an efficiency of 106.19%. Increasing primer concentrations were also tested using template RNA incubated in 1:1 in fresh media/2.5% IGEPAL-630 diluted 1:5 in NF-H₂O to mimic diluted lysate, containing 10U/ml RNasin. (Figure 2G continued)

Lower percentage of lysate in the reaction increases sensitivity and accuracy of direct lysis RT-qPCR

In order to test the effect of increased amounts of lysate on the direct lysis RT-qPCR, the addition of 2 or 3µl of VPM/2.5% IGEPAL-630 lysate to a 10µl reaction, corresponding to 20 or 30% of the reaction volume, was tested. However, this significantly decreased reaction efficiency and sensitivity. (Figure 2H)

246 Previous results showed that the VPM lysate contained RT-qPCR inhibitors. We therefore tested
 247 whether diluting out the lysate in the reaction could improve accuracy of quantification and sensitivity
 248 compared to equivalent amounts of vRNA. To reduce pipetting errors, VPM lysate was diluted in NF-
 249 H₂O prior to addition to the RT-qPCR reaction. Whilst VPM lysate at 10% reaction volume showed a Ct
 250 difference of 1.52 cycles (± 0.78 SEM) compared with equivalent vRNA, lowering concentration of lysate
 251 reduced this further to < 1 cycle: 2%, 0.47 cycles (± 0.43 SEM); 1%, 0.39 cycles (± 0.38 SEM); and 0.5%,
 252 0.46 cycles (± 0.38 SEM). Overall, decreasing volumes of VPM lysate in the reaction showed significant
 253 improvements in sensitivity and accuracy when compared to equivalent amounts of vRNA and at 2%,
 254 1%, and 0.5% of the total reaction volume there was no significant difference between vRNA and lysate
 255 of equivalent amounts. (Figure 2H)

DISCUSSION

In this study, we show a direct lysis protocol for a one-step RT-qPCR of SARS-CoV-2 in cell culture supernatant, achieving Ct results within one cycle of those obtained using traditional viral RNA purification, whilst inactivating SARS-CoV-2.

The best performing primer pair in our protocols, CDC N3, is designed to recognize all currently known clade 2 and 3 viruses of the *Sarbecovirus* subgenus. It is therefore likely directly applicable to other viruses of this subgenus and, with changes in primers, to other CoVs. Our results once again show the importance of testing for the optimal concentration of PCR primers and checking for primer dimer formation, particularly when using fluorescent dye incorporation RT-qPCR. We found that CDC N1, DZIF RdRp, and to a lower extent DZIF N show primer dimers at relatively low primer concentrations. We found that DZIF N forms multimers of its product as observed on the agarose gel evaluation and confirmed by Sanger sequencing, which could affect the efficiency and results from probe-based RT-qPCR diagnostic assays.

As mentioned in the introduction, other groups have tested the use of detergents in SARS-CoV-2 patient swabs; using 1% Triton X-100 or 1% Tween-20 increased Ct values of patient samples, which may increase in comparison to RNase inhibitor-treated samples, in (Pearson et al., 2021), while others found no significant difference in Ct but a decrease in fluorescence intensity / efficiency using Triton X-100 up to 7%/reaction or Tween-20 up to 15%/reaction (Smyraki et al., 2020). This reflects our observation for IGEPAL-630.

This direct lysis protocol offers good starting point to develop similar protocols for other enveloped virus direct RT-qPCR since 1% Triton X-100 and 5% Tween-20 show highly promising results with good sensitivity. Modifying the percentage of detergent, especially for Tween-20, will likely improve results. This is particularly interesting since 0.5% Triton X-100, which is the final concentration in a 1:1 lysis buffer/VPM mix, has been shown to completely inactivate SARS-CoV-2 (PHE, 2020b). Tween-20 will need to be used at higher concentrations since live virus can still be recovered from 30min treatment with 0.5% final concentration Tween-20 (PHE, 2020a).

Others found a proteinase K digest prior to heat lysis to be beneficial to detecting SARS-CoV-2 in patient samples (Genoud et al., 2021; Nique et al., 2021). In our hands, heat lysis of SARS-CoV-2 VPM gave significantly worse results than using a lysis buffer, therefore that avenue was not pursued further for *in vitro* samples. The addition of a proteinase K digest worsened the sensitivity of the RT-qPCR for 0.25% IGEPAL-630, 1% Triton X-100, or 5% Tween-20/VPM and /vRNA. This may be due to the prolonged incubation time, the presence of EDTA, added to protect RNA stability during heat inactivation of the proteinase K, impairing the RT-qPCR reaction, or heat degradation of the RNA.

One of the limiting factors in direct lysis RT-qPCR is that the volume of lysate, and thereby vRNA copies, added to the reaction is limited. However, the direct lysis protocol was found to be sensitive down to <0.0043 TCID₅₀/reaction and showing a 1E6 dynamic range, which should be more than sufficient for *in vitro* experiments.

We found that 2.5% IGEPAL-630 lysis buffer concentration, 1.25% final concentration, slightly decreases the reaction efficiency but does not affect the sensitivity of the RT-qPCR. However, reaction sensitivity was strongly affected by fresh cell culture media. The addition of as little as 20U/ml RNasin rescued the decrease. This is in agreement with the findings of Pearson et al, who also found that adding RNaseOUT RNase inhibitor significantly increased the sensitivity of their direct RT-qPCR of SARS-CoV-2 patient swab samples, achieving Cts only 3 cycles higher than using RNA purification and RT-qPCR (Pearson et al., 2020). This suggests that a broad range of RNase inhibitors can be used in direct lysis assays.

With very low amounts of template RNA, 18.9/reaction, it was observed that 2.5% IGEPAL/NF-H₂O mix showed a decrease of around 3.69Cts without RNasin, indicating a small contamination with RNases in the lysis buffer and/or small amounts in NF-H₂O since the addition of RNasin rescued the reduction in an 2.5% IGEPAL/fresh cell culture media mix even beyond the NF-H₂O control. It shows that despite careful handling, working in PCR cabinets, and decontamination of surfaces, RNase contaminations can occur easily and may not show at higher concentration RNA. It is therefore further recommended to add RNases to the lysis buffer.

Interestingly, the addition of RNase inhibitors had no impact on the 2.5% IGEPAL/VPM lysis. This is either due to the much higher amount of RNA present, an estimated roughly 10,000 more, so that the impact of small amounts of RNases may not be visible. Similarly, viral RNA could still be loosely associated with nucleocapsid proteins, protecting its degradation by RNases.

Whilst SARS-CoV-2 overall only contains around 38% of G and C nucleotides combined, the N region is one of the most GC-rich areas of the virus at 47% with some stretches reaching over 60% GC-content. However, the lack of effect of the addition of betaine suggests that there doesn't seem to be any PCR inhibition by those areas. Whilst previous work shows that betaine concentrations of up to 2M can improve PCR efficiency (Jensen et al., 2010), we found 1M betaine to strongly decrease the efficiency of the RT-qPCR reaction. A reduction of PCR efficiency by betaine was observed by (Zhang et al., 2009) who recommend the addition of ethylene glycol or 1,2-propanediol to improve amplification of regions with higher GC contents. We did not pursue this avenue further, since dilution of lysates showed that RT-qPCR inhibitors rather than a lack of RNA accessibility or virion lysis were the cause of decreased sensitivity.

Looking at our combined results thus far, we found that IGEPAL-630 on its own was not reducing RT-qPCR sensitivity. Fresh cell culture media decreased sensitivity but this was restored by the addition of RNase inhibitors. However, 2.5% IGEPAL-630/VPM lysate was still showing sensitivities 1.52 (± 0.78 SEM) Cts lower than extracted vRNA when added at 10% reaction volume. Testing lower percentage reaction volumes shows that the sensitivity difference can be reduced to less than 1 Ct when lysate is diluted out. This indicates that it is not a lack of complete virus lysis but the presence of RT-qPCR inhibitors in the virus infection media that is limiting the sensitivity.

In conclusion, our method allows inactivation and direct RT-qPCR of SARS-CoV-2 in cell culture medium without the need for heat inactivation or proprietary ingredients. This allows for the fast, efficient, and cost effective analysis of *in vitro* SARS-CoV-2 experiments, quantifying the entire replication cycle, including release, of the virus. For highest sensitivity (lowest Ct values), we recommend the use of 10% lysate of the final volume of the RT-qPCR reaction. For highest accuracy, compared to extracted vRNA,

addition of 1-2% lysate in the final reaction volume is recommended. Since these are low pipetting volumes, a prior dilution step in NF-H₂O can reduce pipetting errors. (Figure 3A)

The direct lysis RT-qPCR protocols allows for the use of clinical samples without the need for recombinant reporter viruses, allowing for quick screening of new variant strains. It measure the entire replication cycle, including virus release, within a single-step measurement. This increases speed, reduces costs, and improves the ability to identify a broader range of antiviral agents and host genes involved in the replication cycle. Results can be obtained in 2.5-3h from culture harvest, increasing speed over second round infections or titration assays significantly. (Figure 3B)

We have applied this method to a variety of applications so far, including high-throughput screening, assessing the impact of drugs and other inhibitors on SARS-CoV-2 production, quantifying viral titers, both directly and using endpoint titration . The applications *in vitro* are nearly endless and can be adapted to most virological questions. It is likely, that this method can easily be adapted to most other enveloped viruses. (Figure 3C)

MATERIALS AND METHODS

Reagent or Resource	Source	Identifier
Bacterial and virus strains		
All SARS-CoV-2 patient isolates are described in the Cells & viruses section	N/A	N/A
10-Beta	NEB/in-house	#C3019H
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium	Sigma-Aldrich	Cat#D6171
Heat-inactivated Fetal Bovine Serum	Gibco	Cat#10500064
Ultraglutamine-I	Lonza	Cat#BE17-605E/U1
MEM Non-essential Amino Acids	Lonza	Cat#BE13-114E
Penicillin-Streptomycin (10,000 U/mL)	Gibco	Cat#15140148
Trizma hydrochloride solution (pH 7.5, 1M)	Sigma-Aldrich	Cat#T2319
IGEPAL CA-630 for molecular biology	Sigma-Aldrich	Cat#I8896
Triton X-100	Sigma-Aldrich	Cat#T8787
Tween 20	Sigma-Aldrich	Cat#P9416
RNase-free NaCl (5M)	Thermo Fisher Scientific	Cat#AM9760G
Nuclease-free water	Qiagen	Cat#129114
Betaine solution (5M)	Sigma-Aldrich	Cat#B0300
Proteinase K	Qiagen	Cat#19133
EDTA (0.5M)	Sigma	Cat#03690
RNasin Plus RNase Inhibitor	Promega	Cat#N2611
UltraPure Agarose	Invitrogen	Cat#16500500
SYBR Safe DNA Gel Stain	Invitrogen	Cat#S33102
Critical Commercial Assays		
GoTaq 1-Step RT-qPCR System	Promega	Cat#A6020
mMessage mMachine SP6 transcription kit	Invitrogen	Cat#AM1340
Qubit RNA BR Assay Kit	Invitrogen	Cat#Q10211
Tapestation RNA Screen Tape, Ladder & Sample Buffer	Agilent	Cat #5067-5576, -5577, and -5578
Zero Blunt TOPO PCR cloning Kit	Invitrogen	Cat#450245
QIAamp Viral RNA Mini kit	Qiagen	Cat#52906
QIAprep Spin Miniprep Kit	Qiagen	Cat#27106
Pierce Protein Concentrator, 100K MWCO	Thermo Scientific	Cat#88524
Experimental models: Cell lines		
Vero E6	ATCC	Cat#CRL-1586
Oligonucleotides		
All the DNA oligonucleotides are listed in Table S2	Life Technologies	N/A
Recombinant DNA and RNA		
All recombinant gene fragments are described in Table S1	IDT Invitrogen	N/A

All the RNA fragments produced by <i>in vitro</i> transcription are described in Table S1	N/A	N/A
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Software and algorithms

GraphPad Prism 9.2.0	GraphPad Software	N/A
LinRegPCR	N/A	https://medischebiologie.nl/files/ab-1513875668-1-15
LC480Conversion	N/A	https://medischebiologie.nl/files/ab-1513875668-1-15
LightCycler480 software 1.5	Roche	Cat#04994884001

Resource availability

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Christine Tait-Burkard (Christine.burkard@roslin.ed.ac.uk)

Experimental model and subject details

Cells and viruses.

Vero E6 (ATCC CRL-1586) cells were maintained as monolayer cultures in Duplecco's modified Eagle's medium (DMEM, Sigma), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS, Gibco), 1X Ultraglutamine-I (Lonza), 1X Non-essential Amino Acids (NEAA, Lonza), and 1x Penicillin-Streptomycin (Gibco) at 37°C in a 5% CO₂ atmosphere.

Samples from confirmed COVID-19 patients were collected by a trained healthcare professional using combined nose-and-throat swabbing. The sample was stored in virus transport medium prior to cultivation and isolation on Vero E6 (ATCC CRL-1586) cells following sterile filtration through a 0.1µm filter. Samples were obtained anonymized by coding, compliant with Tissue Governance for the South East Scotland Scottish 279 Academic Health Sciences Collaboration Human Annotated BioResource (reference no. SR1452). Virus sequence was confirmed by Nanopore sequencing according to the ARCTIC network protocol (<https://artic.network/ncov-2019>), amplicon set V3, and validated against the patient isolate sequence. The main virus isolate used in this project was EDB-2 (Scotland/EDB1827/2020, UK lineage 109, B1.5 at the time, now B.1). Methods were also confirmed using EDB-1, EDB-B117-2 (alpha variant), and EDB-B16172-1 (delta variant).

Primer optimization using *in vitro* transcribed RNA fragments.

Fragments of the SARS-CoV-2 reference sequence Wuhan-Hu-1 NC_045512.2 were purchased as double-stranded DNA fragments. The fragments don't correspond to full genes as they were designed to work for fragment rather than gene synthesis. Fragment 10 (IDT), containing parts of RdRp (nsp12, orf1ab) and fragments 18 (IDT) and 19 (Life Technologies) (Supplementary Table S1), containing parts of N (orf10) were cloned into the pCR-Blunt-II vector using the PCR Blunt Topo kit according to the manufacturer's instructions and transformed into 10-beta CaCl₂-chemically competent bacteria (originally NEB, generated in-house). Following amplifications of colonies and purification of plasmids using a QiAprep Mini kit (Qiagen), orientation of the gene fragment was determined by restriction digest. Following determination of correct forward orientation, plasmids were linearized using the NotI-HF (NEB) restriction site and transcribed *in vitro* using the mMessage mMachine SP6 (Invitrogen) transcription kit according to the manufacturer's instructions. Transcribed RNA quality and quantity was determined using a NanoDrop 1000 spectrophotometer, the Qubit BR RNA Assay kit, and capillary electrophoresis using a Tapestation and the RNA screentape, all according to the manufacturer's instructions.

To test primer efficiency, template RNA (tempRNA) 10 was used for DZIF RdRp, tempRNA 18 for CDC N1 & N3, and DZIF N, and tempRNA 19 for CDC N2 primers, respectively. A list of the primer sequences can be found in Supplementary Table S2. 10-fold serial dilutions of the template RNA were generated in NF-H₂O and primers tested using 1µl of a 1E-6 dilution in a 10µl reaction, corresponding to 699 copies of tempRNA 10, 1890 copies of tempRNA 18, and 699 copies of tempRNA 19. A series of concentrations from 50-600nM in symmetric and asymmetric forward and reverse primer concentrations were added to the RT-qPCR reaction using the GoTaq 1-Step RT-qPCR System (Promega) according to the manufacturer's instructions using the standard annealing temperature of 60°C. RT-qPCR was run on a LightCycler480 and analyzed using the corresponding software and LinRegPCR.

RT-pPCR products were analyzed on a 2% Agarose (Invitrogen) gels using SYBR Safe DNA gel stain (Invitrogen) according to the manufacturer's instructions. Larger DZIF N product fragments were

excised and purified using the QIAquick Gel Extraction kit (Qiagen) and analyzed by Sanger sequencing using the DZIF N forward and reverse primers, respectively.

To obtain absolute reaction efficiencies, 10-fold serial dilutions of template RNA were added to the RT-qPCR reaction (as described above). A semilog fit was performed using Graphpad Prism to determine the slope to determine the RT-qPCR efficiency.

Detergent lysis buffer method optimization

Lysis buffers were made up using 5M NaCl and 1M Tris-HCl pH7.5 stock solutions to a final concentration of 150mM NaCl, 10mM Tris-HCl pH7.5 and supplemented with final concentrations of a range of concentrations of IGEPAL CA-630, 1 or 10% of Triton X-100, or 5% Tween-20 in nuclease-free water, supplemented with RNasin Plus as indicated in individual experiments.

Virus production media (VPM) were generated by inoculating near-confluent Vero E6 cells at MOI 0.1 of EDB-2, EDB-1, or EDB-B117-2 (as determined by endpoint titration on Vero E6 cells – data shown are for EDB-2) for 1.5h before change of media to complete media and infection for 36h. Supernatant was harvested and debris cleared by centrifugation at 1,500 x g for 10min before freezing in aliquots at -80°C.

For initial optimisation, single-round frozen aliquots of VPM were heat-inactivated at 70°C for 10min in 100µl aliquots in thin-walled PCR tubes using a PCR machine to ensure core temperature was reached for the correct amount of time. After validation of SARS-CoV-2 inactivation, experiments were repeated with non-heat-inactivated VPM. No difference in sensitivity, efficiency, or fluorescence strength could be observed.

vRNA was extracted using the QIAamp Viral RNA Mini kit according to the manufacturer's instructions and added to the RT-qPCR reaction at equivalent volumes and dilutions corresponding to the amount VPM added as VPM/lysis buffer to the reaction.

Virus lysis buffer, containing the indicated amounts of detergent and additives was thoroughly mixed by pipetting at a 1:1 ratio with VPM and incubated for 20 minutes at room temperature, unless otherwise

indicated. Different incubation times were tested and whereas shorter incubation times increased Ct values, no improvement was observed after 20 minutes.

Unless otherwise indicated, RT-qPCR reactions using the GoTaq 1-Step RT-qPCR reactions were set-up in 10µl final reaction volumes according to the manufacturer's instructions containing 350nM CDC N3 primer of each, forward and reverse primer and VPM/lysis buffer mix was added at 10% final volume. No further reference dye was added. The RT-qPCR was run according to the manufacturer's instructions on a LightCycler480 using an annealing temperature of 60°C and analyzed using the corresponding software and LinRegPCR.

Proteinase K treatment

To test the addition of proteinase K to the lysis buffer, each detergent lysis buffer was supplemented with 0.1AU/ml proteinase K (Qiagen) and 0.83mM final concentration EDTA (Sigma) to prevent heat damage to RNA during heat inactivation. After mixed 1:1 with VPM or vRNA in nuclease-free water (NF-H₂O) samples were incubated in thin-wall tubes in a PCR machine for 30min at 56°C prior to heat inactivation for 10min at 95°C. The inactivated lysate was added at 10% final volume to the RT-qPCR reaction; set-up and analyzed as described before.

Efficiency curves

10-fold serial dilutions of template RNA or VPM in mock lysate with NF-H₂O or media were generated as described for the respective experiment. Lysate dilutions were added at 10% final volume to the RT-qPCR reaction; set-up and analyzed as described before. A semilog fit was performed using Graphpad Prism to determine the slope to determine the RT-qPCR efficiency.

RNasin and betaine addition experiments

Lysis buffer was supplemented with RNasin Plus (Promega) as indicated, using a stock concentration of 40,000U/ml. Lysis and RT-qPCR reaction were performed as described above.

Betaine (Sigma) was added to the RT-qPCR reaction to the final concentration stated, and RT-qPCR reaction and analysis performed as described above.

Virus inactivation

VPM was mixed 1:1 with lysis buffer or PBS and incubated for 20min at room temperature. A 10-fold serial dilution of lysate was made in inoculation medium, and near-confluent Vero E6 cells inoculated with the diluted virus. Inoculum was removed 1.5 hours post inoculation and replaced with culture medium. At 48hpi supernatant was harvested and heat-inactivated at 70°C for 10min before RNA extraction using the QIAamp Viral RNA Mini kit or lysis of the supernatant before quantification of viral RNA. Due to the toxicity of the lysis buffer mix on cells, half of the lysate/VPM or lysate/PBS mixtures were subjected to a buffer exchange using a 100kDa MWCO protein concentrator (Pierce, Thermo Scientific), reduction to 20% volume and two washes with PBS prior to serial dilution and inoculation of Vero E6 cells as described above.

Viral RNA or lysates were subjected to RT-qPCR analysis using 350nM of CDC N3 primers in the GoTaq 1-Step RT-qPCR System according to the manufacturer's instructions and as described above for lysates. If the Ct recorded was 35 or above, a well was classified as non-infected TCID₅₀ calculated accordingly.

Recommended final protocol for direct lysis RT-qPCR

Inside containment level 3 facilities, within an appropriate biosafety cabinet, and according to local health and safety guidelines, mix equal amounts of virus-containing supernatant with virus lysis buffer (VL buffer - 2.5% IGEPAL CA-630, 150mM NaCl, in 10mM Tris-Cl, pH7.5) by thoroughly pipetting up and down. Incubate the mixture at room temperature for 20min before decontamination of the containment tube and removal from high containment facilities, according to local health and safety guidelines. Agitation on a plate shaker or equivalent can improve lysis efficiency, especially in small volume containers, such as 96- or 384-well plates.

Prepare stocks of equal amounts of CDC N3 forward and reverse primers to 35mM concentration each; this minimizes the volume of primer to add the reaction. Each 10µl reaction is made up of 0.1µl primer mix, 0.2µl RT mix, and 5µl 2x qPCR master mix, leaving 4.7µl for the template. To minimize pipetting errors, we recommend diluting the lysate 1:4.7 or 1:5 in NF-H₂O before adding 4.7µl for the diluent to the reaction. If higher accuracy is desired, a lower percentage lysate and consequently a higher dilution can be prepared or a lower amount added to the reaction. Mixing can be done by pipetting up and down or, following sealing of the plate or PCR tubes, lysates can be briefly spun down, mixed on a plate shaker or equivalent then spun down again. Samples are run according to the GoTaq 1-Step RT-qPCR System protocol using a 60 °C annealing temperature.

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AUTHOR CONTRIBUTIONS

Outlined the study NC, SF, AD, and CTB; performed experiments NC, SF, and AD; analyzed data NC, AW, and CTB; interpreted data NC, SF, AD, AW, and CTB; wrote manuscript, NC and CTB, with contribution of other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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FIGURE LEGENDS

Figure 1

Primer selection for 1-step-RT-qPCR amplification of SARS-CoV-2. Increasing symmetric (single number) and asymmetric (forward/reverse separated) concentrations of forward and reverse primer pairs were tested in a 1-Step-RT-qPCR reaction using templates representing the respective target regions. Primer sets CDC N1, N2, and N3, and DZIF N and RdRp (from left to right) were tested using 699 (CDC N2 and DZIF RdRp) or 1890 (CDC N1 and N3, and DZIF N) template copies per reaction. A) shows the amplification curves of selected primer concentrations. Exact concentrations for each curve are listed in the legend for each graph. Continuous lines represent symmetric concentrations whilst dash/dot curves show asymmetric primer concentrations. Grey curves depict no-template reactions. B) shows the melting curves (72-95°C) of the PCR reaction with the highest shown symmetric, and if shown in A), asymmetric concentration. C) shows the Ct values as calculated using the LightCycler software using the 2nd derivative of the max calculation. Efficiencies for each amplification were calculated using LinRegPCR. n=2; curves represent the average. D) PCR products obtained were analyzed on an agarose gel to assess primer-dimer formation in high and low template (indicated above gel pictures) RT-qPCR reactions. E) Serial dilutions of template were run in RT-qPCR reactions to assess efficiency of the PCR reactions for the best performing primer pairs. A semilog fit curve was calculated using GraphPad prism to assess reaction efficiency from the slope. Samples excluded from the efficiency calculation are greyed out, as they were beyond reaction sensitivity limits. n=3*2, error bars represent min and max.

Figure 2

Direct lysis RT-qPCR. Virus production medium (VPM), corresponding amounts of vRNA, or template RNA (as indicated in individual experiments) were lysed 1:1 with detergent lysis buffer for 20min at room temperature before addition to a 1-Step-RT-qPCR reaction at 10% of the reaction volume using 350nM symmetric concentrations of the CDC N3 pair, unless otherwise indicated. Ct values as calculated using

the LightCycler software using the 2nd derivative of the max calculation. Efficiencies for each amplification were calculated from amplification curves using LinRegPCR. For serial dilutions, a semilog fit curve was calculated using GraphPad Prism to assess reaction efficiency from the slope.

A) Different detergents, 0.25% IGEPAL-630 (I630), 10% and 1% Triton X-100, and 5% Tween 20 (Tw20) were assessed for their ability to release vRNA from cell supernatant and use in a direct lysis RT-qPCR. In parallel to vRNA and a standard lysis of 1:1 VPM:lysis buffer, vRNA and VPM were lysed 1:1 and incubated for 30min at 56°C in lysis buffers containing 0.1AU/ml proteinase K (PK) and 0.83mM EDTA before heat inactivation for 10min at 95°C. n=2. B) Increasing concentrations of IGEPAL-630 were tested in the direct lysis reaction in comparison to equivalent amounts of vRNA in NF-H₂O. n=2 C) Template RNA in NF-H₂O was lysed in buffer containing 2.5% IGEPAL-630. n=2 D) A serial dilution of template RNA in NF-H₂O or fresh cell culture media was incubated in lysis buffer containing 2.5% IGEPAL-630. n=2, error bars represent min and max. E) 18.9 RNA template copies were incubated in lysis buffer containing 2.5% IGEPAL-630 and increasing amounts of RNasin Plus (stock concentration 40U/μl) or fresh media or NF-H₂O. Similarly, VPM was incubated using a low or a high amount of RNasin in the lysis buffer, and compared to vRNA extracted from an equivalent amount of VPM. n=2 F) An RT-qPCR reaction was set up containing 10% either VPM lysate using 2.5% IGEPAL-630 or vRNA in NF-H₂O. Reactions were supplemented with 0.1, 0.5, or 1M betaine. n=2 G) Increasing symmetric concentrations of CDC N3 primer were added to the VPM/lysate RT-qPCR reaction. n=2. Serial dilutions of VPM in lysis buffer or template RNA in mock lysis buffer were run in an RT-qPCR assay and analyzed as described above. n=3*2. H) Decreasing and increasing amounts of VPM lysate were added to the RT-qPCR reaction, corresponding to 0.5-30% of the reaction volume (as indicated for each curve). Corresponding amounts of vRNA in NF-H₂O were run in parallel. Curves represent an average of 3*2, except for 20- and 30%, representing an n=2. Ct values n=3*2 show mean +/- SEM and were analyzed using a 2-way ANOVA. ** P<0.005 (0.0012)

Figure 3

Direct lysis RT-qPCR method. A) depicts an overview for using the method. B) highlights the advantages of using direct lysis RT-qPCR. C) shows applications for which direct lysis was successfully used and suggestions for other applications. *Direct measurement of virus titers may not accurately represent infectious virus dependent on harvesting time and conditions. Similarly, growth curves at later points of infection may not accurately represent infectious virus particles present in the solution. Created with <https://biorender.com/>.

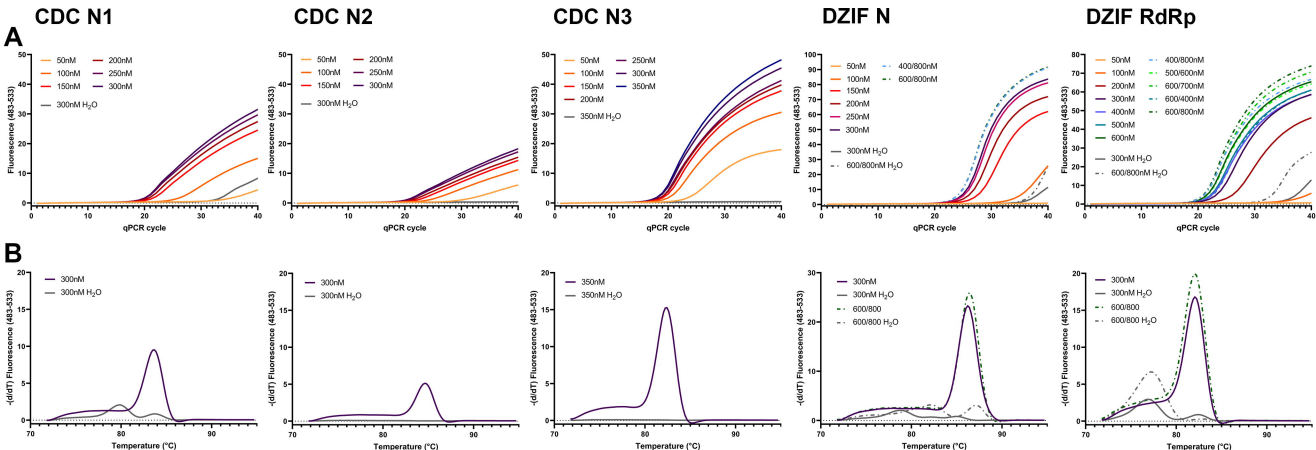
TABLES

Table 1: Virus inactivation following a 20-minute incubation of virus stock diluted 1:1 with 0.25 or 2.5% IGEPAL-630, or PBS, with or without buffer exchange.

	Mean virus titer in log ₁₀ TCID ₅₀ /ml [95% confidence interval]	Titer reduction in log ₁₀ TCID ₅₀ /ml [95% confidence interval]
PBS 1:1 (neat)	7.05 [6.75-7.22]	-
0.25% I-630 1:1 (neat)	≤2.8*	≥4.25 [3.95-4.42]
2.5% I-630 1:1 (neat)	≤2.8*	≥4.25 [3.95-4.42]
PBS-treated 1:1 (buffer exchange)	6.90[6.34-7.14]	-
0.25% I-630 1:1 (buffer exchange)	2.71 [2.59-2.81]	4.35 [4.05-4.52]
2.5% I-630 1:1 (buffer exchange)	≤0.8*	≥6.25 [5.95-6.42]

* Cytotoxicity in undiluted, 1E-1, and 1E-2 dilutions. Limit of detection for test was 2.8 log₁₀ TCID₅₀/ml. 95% confidence interval cannot be calculated

*Limit of detection for test was 0.8 log₁₀ TCID₅₀/ml. 95% confidence interval cannot be calculated



C

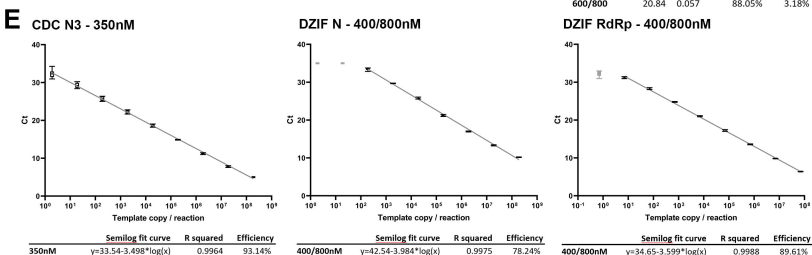
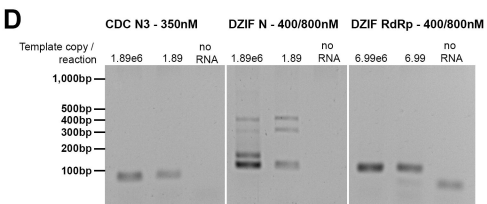
Primer conc (nM)	Average Ct	Stdev	Efficiency	Av Efficiency	Efficiency (LinRegPCR)	Stdev
50	35 (max)	n/d	26.95%	1.34%		
100	25.86	0.077	67.55%	6.15%		
150	21.22	0.030	77.80%	2.97%		
200	20.47	0.063	78.90%	0.71%		
250	19.87	0.014	80.55%	2.47%		
300	19.80	0.004	81.25%	4.31%		

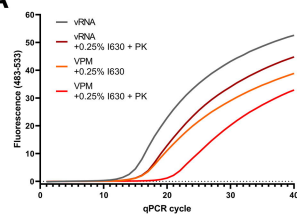
Primer conc (nM)	Average Ct	Stdev	Efficiency	Av Efficiency	Efficiency (LinRegPCR)	Stdev
50	30.09	0.140	45.60%	2.55%		
100	24.06	0.183	68.60%	10.32%		
150	22.02	0.004	58.30%	18.95%		
200	20.93	0.10	76.00%	1.13%		
250	20.35	0.005	81.40%	4.67%		
300	20.03	0.041	73.40%	5.52%		

Primer conc (nM)	Average Ct	Stdev	Efficiency	Av Efficiency	Efficiency (LinRegPCR)	Stdev
50	23.57	0.144	61.60%	3.39%		
100	20.33	0.212	76.80%	0.42%		
150	19.46	0.002	83.80%	0.00%		
200	18.98	0.073	83.60%	1.13%		
250	18.60	0.131	87.90%	0.28%		
300	18.50	0.138	87.90%	0.28%		
350	18.21	0.023	93.40%	7.50%		

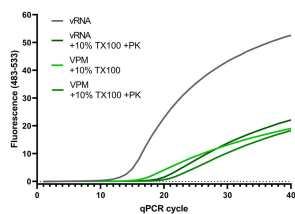
Primer conc (nM)	Average Ct	Stdev	Efficiency	Av Efficiency	Efficiency (LinRegPCR)	Stdev
50	n/d	n/d	n/d	n/d		
100	35 (max)	n/d	55.65%	2.33%		
150	27.67	0.655	74.45%	1.06%		
200	26.17	0.017	79.05%	0.07%		
250	25.47	0.055	80.60%	1.14%		
300	25.06	0.109	82.55%	0.07%		
350	25.07	0.016	83.10%	1.98%		
400	24.88	0.134	85.00%	1.41%		
500	24.81	0.010	84.05%	0.35%		
600	24.62	0.003	84.35%	0.35%		
400/800	24.33	0.173	85.20%	1.41%		
600/800	24.29	0.289	85.05%	1.20%		

Primer conc (nM)	Average Ct	Stdev	Efficiency	Av Efficiency	Efficiency (LinRegPCR)	Stdev
50	n/d	n/d	n/d	n/d		
100	35 (max)	n/d	40.60%	1.84%		
150	28.23	0.012	60.95%	0.07%		
200	26.00	0.047	68.10%	0.71%		
250	24.17	0.011	74.00%	0.85%		
300	23.24	0.077	76.40%	0.57%		
350	22.75	0.046	79.10%	1.27%		
400	22.19	0.027	79.50%	0.57%		
450	21.75	0.001	81.20%	2.26%		
500	22.00	0.126	80.20%	0.99%		
600	21.34	0.005	85.15%	0.35%		
600/400	22.42	0.089	82.70%	2.12%		
500/600	21.51	0.019	83.95%	2.76%		
400/900	20.82	0.055	85.15%	0.64%		
600/700	20.96	0.025	89.90%	3.39%		
600/800	20.84	0.057	88.05%	3.18%		

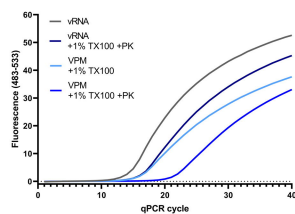


A

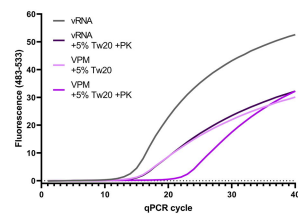
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
vRNA	13.53	0.1393	86.56%	1.15%
+0.25% I630 +PK	14.75	0.059	87.34%	1.99%
Virus prod. media	14.89	0.160	86.77%	1.38%
+0.25% I630	19.70	0.015	83.29%	0.99%



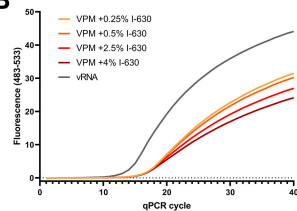
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
vRNA	13.53	0.140	86.56%	1.15%
+10% TX100 +PK	19.39	0.616	74.41%	9.43%
Virus prod. media	16.80	0.062	59.20%	6.23%
+10% TX100 +PK	20.88	0.753	62.89%	3.42%



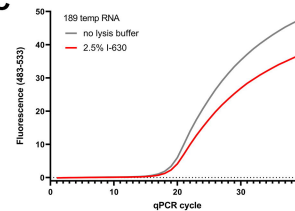
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
vRNA	13.53	0.139	86.56%	1.15%
+1% TX100 +PK	14.90	0.209	82.94%	4.43%
Virus prod. media	15.01	0.163	80.16%	0.34%
+1% TX100 +PK	20.35	0.201	82.94%	1.09%



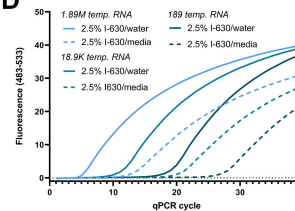
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
vRNA	13.53	0.139	86.56%	1.15%
+5% Tween20 +PK	14.77	0.016	83.89%	2.48%
Virus prod. media	14.94	0.068	78.46%	0.71%
+5% Tween20 +PK	21.57	0.015	87.37%	3.03%

B

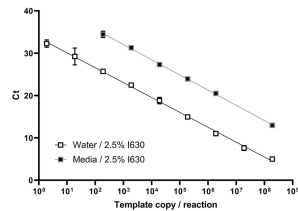
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
vRNA	13.79	0.003	84.15%	2.82%
Virus prod. media	16.64	0.010	83.16%	3.65%
+0.25% I630	16.55	0.071	80.00%	1.30%
+0.5% I630	16.24	0.037	79.41%	6.62%
+2.5% I630	17.16	0.126	77.35%	0.03%

C

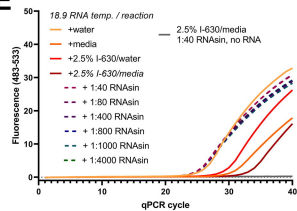
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
189 RNA templates / reaction	18.41	0.382	91.20%	5.30%
+2.5% I630/water	18.82	0.083	81.70%	4.38%

D

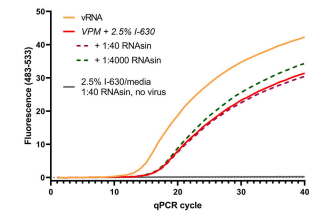
RNA templates (copy/reaction)	Average Ct	Stdev	Media/2.5% I630	Stdev
18,900,000	5.00	0.000	12.97	0.038
1,890,000	7.62	0.064	20.50	0.025
189,000	11.01	0.040	23.94	0.282
18,900	14.96	0.013	27.33	0.004
1,890	18.82	0.086	31.28	0.058
189	22.49	0.034	34.47	0.749
18.9	25.71	0.040	n/d	
1.89	29.23	0.222	n/d	



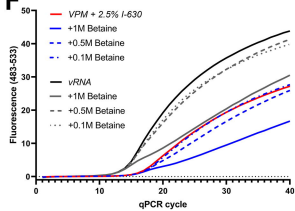
Sample	Semilog fit curve	R squared	Efficiency
2.5% I630/water	$y=33.60-3.316*\log(x)$	0.9985	92.49%
2.5% I630/media	$y=42.82-3.588*\log(x)$	0.9984	89.98%

E

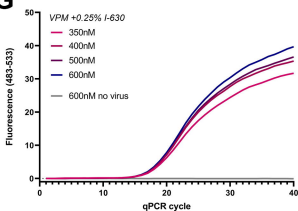
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
18.9 RNA templates/reaction	26.87	0.030	89.00%	1.84%
+ water	29.41	0.309	87.00%	2.83%
+ media	30.56	0.780	89.55%	5.30%
+2.5% I630/water	33.16	0.370	88.70%	2.83%
+2.5% I630/media	24.95	0.047	91.25%	0.78%
+1.40 RNAsin	25.03	0.038	95.85%	2.47%
+1.80 RNAsin	24.89	0.038	90.95%	0.49%
+1.800 RNAsin	24.96	0.096	89.15%	0.07%
+1.1000 RNAsin	24.99	0.146	91.40%	1.27%
+1.4000 RNAsin	24.95	0.037	89.05%	1.63%



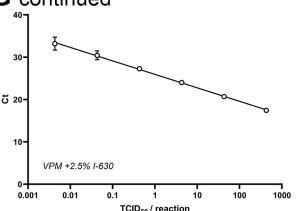
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
vRNA	13.71	0.001	86.50%	3.68%
Virus prod. media	16.30	0.023	89.85%	7.14%
+2.5% I630	16.42	0.302	93.30%	4.67%
+1.40 RNAsin	16.34	0.095	90.40%	6.51%

F

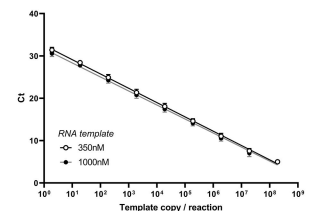
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
vRNA	13.76	0.120	83.53	1.00
+1M Betaine	12.17	0.009	72.00	2.97
+0.5M Betaine	13.17	0.073	84.38	4.57
+0.1M Betaine	13.64	0.024	87.65	4.31
Virus prod. media	16.36	0.125	84.07	6.33
+2.5% I630	15.95	0.864	75.00	6.08
+1M Betaine	16.09	0.023	78.75	0.35
+0.5M Betaine	16.72	0.082	85.60	2.26

G

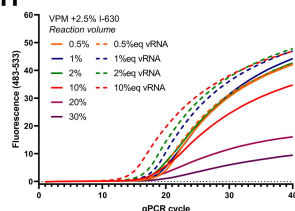
Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
Virus prod. media	15.89	0.071	94.33%	7.64%
+0.25% I630	15.63	0.113	95.80%	4.24%
350nM	15.7	0.085	99.75%	3.11%
400nM	15.465	0.120	101.80%	2.26%

G continued

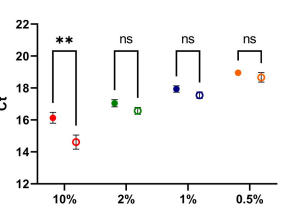
Sample	Semilog fit curve	R squared	Efficiency
350nM	$y=25.95-3.182*\log(x)$	0.9808	106.19%



Sample	Semilog fit curve	R squared	Efficiency
350nM	$y=33.50-3.379*\log(x)$	0.9957	97.67%
100nM	$y=31.65-3.311*\log(x)$	0.9940	100.46%

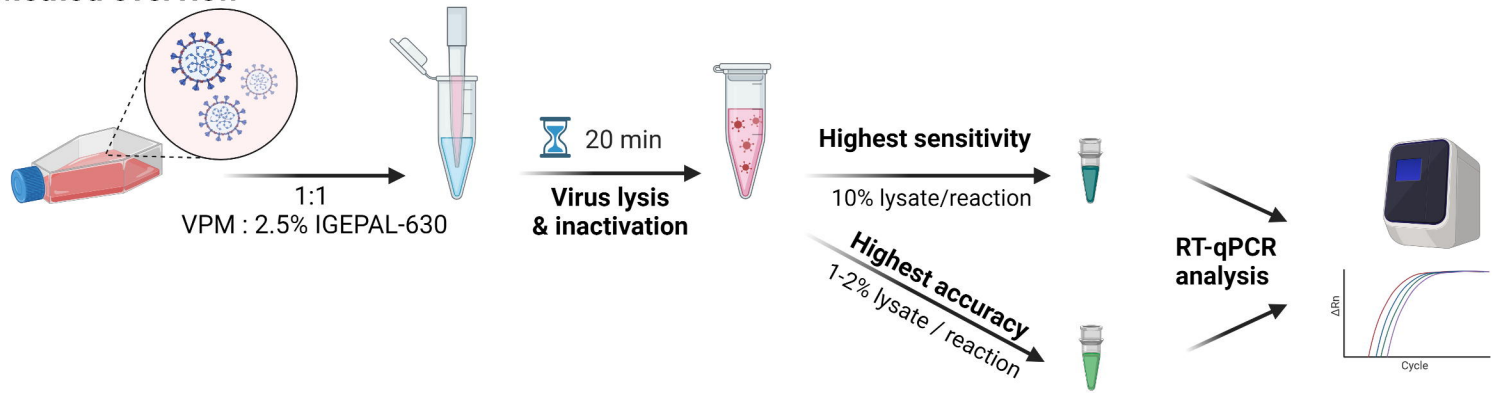
H

Sample	Average Ct	Stdev	Efficiency Av	Efficiency Stdev
Virus prod. Media	19.43	1.084	44.60%	10.30%
+2.5% I630	17.10	0.336	65.95%	2.72%
30% reaction volume	16.13	0.832	82.37%	4.67%
10% reaction volume	17.04	0.553	87.17%	4.08%
2% reaction volume	17.94	0.489	86.26%	3.56%
1% reaction volume	18.95	0.431	91.02%	3.43%
vRNA	14.61	1.081	83.04%	5.54%
+2.5% I630	16.57	0.536	85.63%	1.96%
2% react. vol. equiv.	17.55	0.499	86.41%	4.12%
1% react. vol. equiv.	18.67	0.587	82.16%	4.59%

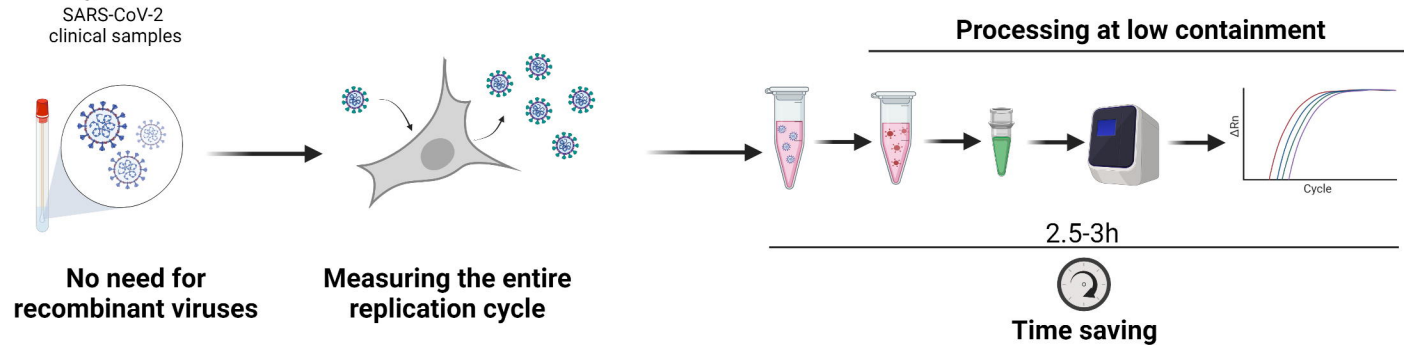


VL buffer in RT-qPCR reaction

A - Method overview



B - Advantages



C - Applications

