bio-protocol

Published: Mar 05, 2022

A Novel PCR-Based Methodology for Viral Detection Utilizing Mechanical Homogenization

Zachary P. Morehouse[1, 2, 3, *], Caleb M. Proctor[2], Gabriella L. Ryan[2] and Rodney J. Nash[3, 4]

[1]Michigan State University College of Osteopathic Medicine, East Lansing, MI, USA
[2]Omni International Inc, A PerkinElmer Company, Kennesaw, GA, USA
[3]Jeevan Biosciences LLC, Tucker, GA, USA
[4]Department of Biology, Georgia State University, Atlanta, GA, USA
*For correspondence: moreho17@msu.edu

Abstract

The impact of viral diseases on human health is becoming increasingly prevalent globally with the burden of disease being shared between resource-rich and poor areas. As seen in the global pandemic caused by SARS-CoV-2, there is a need to establish viral detection techniques applicable to resource-limited areas that provide sensitive and specific testing with a logistically conscious mindset. Herein, we describe a direct-to-PCR technology utilizing mechanical homogenization prior to viral PCR detection, which allows the user to bypass traditional RNA extraction techniques for accurate detection of human coronavirus. This methodology was validated *in vitro,* utilizing human coronavirus 229E (HCoV-229E), and then clinically, utilizing patient samples to test for SARS-CoV-2 infection. In this manuscript, we describe in detail the protocol utilized to determine the limit of detection for this methodology with *in vitro* testing of HCoV-229E.

Keywords: Coronavirus, Virology, PCR, RT-qPCR, Diagnostics, Molecular diagnostics, Homogenize, Infectious disease

This protocol was validated in: Virol J (2020), DOI: 10.1186/s12985-020-01405-y

Background

Polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-qPCR)-based methodologies are the current mainstay of detection for human viral infections (Elnifro *et al*., 2000). These PCR methodologies often rely on multiple chemical extraction steps, for lysis of the viral particles from the clinical sample, followed by a series of washing steps, which are meant to purify the exposed viral genetic material while removing other macromolecules, such as carbohydrates and proteins, that may impact downstream molecular applications (Elnifro *et al*., 2000). Once the purified genetic material has been isolated, it is then placed into the PCR mixture for detection of a specific targeted genetic sequence, thereby verifying the presence of the virus. This traditional extraction model for PCR detection of virus is highly sensitive and specific in confirming infection (Elnifro *et al*., 2000). However, as seen in the global pandemic caused by SARS-CoV-2, the stressed global supply chain could not always ensure availability of the chemical reagents needed for these extractions, driving the need for novel diagnostic techniques (Bustin *et al*., 2020).

Herein, we describe a methodology where mechanical homogenization was used to lyse viral particles, exposing RNA for accurate detection of the virus when placed directly into PCR, without undergoing further extraction steps or chemical washes (Morehouse *et al*., 2020). This methodology has been reported utilizing *in vitro* simulation of clinical viral samples with human coronavirus 229E (HCoV-229E) (Morehouse *et al.*, 2020), as well as in a field validation study with the use of nasopharyngeal and oropharyngeal swabs for the detection of SARS-CoV-2 from patients (Morehouse *et al*., 2021). This homogenization-based direct-to-PCR technique allows for accurate viral detection of human coronaviruses, without the need for time consuming and costly chemical extraction techniques traditionally used in RT-qPCR detection workflows (Morehouse *et al.*, 2020).

In an effort to improve access to cost effective diagnostics solutions, we demonstrate how human coronavirus can be accurately detected in clinical concentrations with similar sensitivity and specificity to the current gold standard extraction-based techniques (Morehouse *et al.*, 2020 and Morehouse *et al*., 2021). It is critical that innovative diagnostic solutions such as this are continually developed with concern for the logistical challenges of providing accurate viral detection in resource-limited settings. Through the utilization of mechanical homogenization, we were able to detect HCoV-229E off spiked swabs, simulating clinically relevant concentrations of virus on nasopharyngeal swabs as an initial proof of concept study for this technique (Morehouse *et al.*, 2021). The homogenization-based direct-to-PCR methodology was then further validated with patient samples screening for SARS-CoV-2 in a later study (Morehouse *et al.*, 2021). In this manuscript, we lay out the detailed protocol utilized in the initial proof of concept testing of our homogenization-based direct-to-PCR technology, utilizing simulated nasopharyngeal swabs with spiked HCoV-229E to determine the limit of detection for this proposed methodology.

Materials and Reagents

1. 2 mL screw cap tubes (Omni International Inc., catalog number: 19-628) prefilled with 1 mL of viral transport media (VTM)
2. Corning 2 mL internal threaded polypropylene cryogenic vials, self-standing with round bottom (Corning, catalog number: 431386)
3. Cotton tipped swabs (Dynarex, catalog number: 4305)
4. PCR tubes: 96 well plate format (Bio-Rad, catalog number: HSP9601) with plate sealing film (Bio-Rad, catalog number: MSB1001)
5. Viral transport media, made following the United States Centers for Disease Control and Prevention (US CDC) protocol (SOP#: DSR-052-05). While we utilized the US CDC VTM made in our laboratory for this protocol, the authors are aware that many other commercially available VTMs are eligible to be substituted based on the availability of reagents in any given laboratory environment.
6. HCoV-229E virus stock in DMEM, prepared from cell culture
   HCoV-229E (ATCC, catalog number: VR-740) cultured following manufacturers recommendations on MRC-5 cells (ATCC, catalog number: CCL-171) in standard DMEM (Gibco, catalog number: 11965092). Cell culture media was collected following manufacturer's instructions 72 h after inoculation, and then utilized as the virus

stock for these experiments.
7. Corning 75 cm^2 U-shaped canted neck cell culture flask with vent cap (Corning, catalog number: 430641U)
8. Corning PureCoat Amine 6-well tissue culture plates (Corning, catalog number: 354721)
9. Luna Universal One-Step RT-qPCR Kit (New England Biolabs, catalog number: E3005S)
10. HCoV-229E N forward and reverse primer set (Integrated DNA Technologies, Custom Oligonucleotide Purchase)
    a. N gene forward primer: 5'-AGGCGCAAGAATTCAGAACCAGAG-3'
    b. N gene reverse primer: 5'-AGCAGGACTCTGATTACGAGAAAG-3'
11. Molecular grade agarose (Bio-Rad, catalog number: 161-3101)
12. Ethidium bromide (Bio-Rad, catalog number: 161-0433)
13. TBE buffer (IBI Scientific, catalog number: IB70153)
14. 100 bp molecular ruler (Bio-Rad, catalog number: 1708202)
15. Trypan blue (Bio-Rad, catalog number: 1450021)

Equipment

1. Bead Ruptor Elite (Omni International Inc., catalog number: 19-042E)
2. CFX connect (Bio-Rad, catalog number: 1855201)
3. Gel Doc EZ Imaging System (Bio-Rad, catalog number: 1708270)
4. Rainin Classic manual pipettes (Rainin, catalog number: 17008708)
5. ThermoFisher Fresco 17 microcentrifuge (Thermo Fisher Scientific, catalog number: 75002421)
6. Gel electrophoresis chamber (BioRad, catalog number: 1704487EDU)
7. Thermo Scientific TSX ultra-low freezer (Thermo Fischer Scientific, catalog number: 09313868)

Software

1. CFX Software Bio-Rad CFX Maestro 1.1 Version 4.1.2433.1219
2. EZ Imager Software Image Lab™ Version 6.0.0 build 25

Procedure

A. HCoV-229E Viral Stock Preparation and Dilutions

1. Grow a viral stock of HCoV-229E on MRC-5 cells and harvest the virus from the cell supernatant, following the manufacturer's instructions. Use the virus to infect 80% confluent MRC-5 cells plated in T-75 flasks with a multiplicity of infection (MOI) of 1.6. Culture the infected cells for another 72 h post-infection, until greater than 70% cytopathic effect (CPE) is observed. Harvest the cell culture supernatant to create the HCoV-229E virus stock for utilization in these experiments. Centrifuge the harvested supernatant at 3,000 × *g* for 10 min, to pellet any cellular debris within the solution. After centrifugation, transfer the supernatant containing the virus stock to a clean 15 mL conical tube for storage and discard the pelleted debris.
2. Following virus stock purification, complete plaque assays to determine a starting viral load. In this protocol, our viral stock had a concentration of 1.2×10^6 PFU/mL of HCoV-229E. Perform plaque assays via serial dilutions of viral stock onto MRC-5 cells plated to 80% confluence in 6-well plates. The starting cell count for this procedure was 200,000 cells/well in 2 mL of media. Following virus addition, allow the cells to grow for 5 days before evaluation of plaque formation.
3. With 10 mL of HCoV-229E viral stock at 1.2×10^6 PFU/mL, dilute the HCoV-229E in a stepwise fashion

by pipetting 1 mL of stock solution into 9 mL of viral transport media, inverting the new dilution three times, and then pipetting 1 mL into the next tube of 9 mL of viral transport media. Conduct this serial dilution until obtaining a stock of 1.2×10^1 PFU/mL. On the final dilution step, following the three inversions of the tube, pipette 1 mL of viral stock and discard it to maintain accurate concentrations. This step was conducted with all reagents thawed to room temperature.

4. Store HCoV-229E stock dilutions in 2 mL cryogenic storage vials at -80°C until needed, if not being utilized within 2 h of stock dilution production.

B. Sample Swab Preparation

1. Thaw HCoV-229E stock dilutions to 4°C, if not currently available for use. Conduct the remainder of the swab preparation procedure at room temperature.
2. Pipette 1 mL of sterile viral transport media (VTM) into clean 2 mL screw cap tubes. Do not lose the caps for these tubes, as they will be needed in the homogenization step.
3. Once HCoV-229E stocks are thawed, prepare swab samples from each dilution for PCR detection by submerging a cotton tipped swab in the HCoV-229E dilution for 5 s, to allow for complete saturation, and then placing the swab directly into one of the prefilled 2 mL screw capped tubes.
4. Break off the stalk of the swab while maintaining the cotton tip within the VTM and replace the screw cap on the 2 mL tube.
5. Repeat steps B2–B4 until the desired number of swabs for each dilution have been created in preparation for PCR detection.

Note: We recommend a minimum of 5 swabs at each concentration for preliminary limit of detection work, with a minimum of 30 additional replicates needed for confirmation once a targeted concentration has been determined.

C. Shaker Mill Homogenization

1. Ensure the screw capped tubes containing 1 mL of VTM and swabs are tightly sealed. Place 2 mL tube carriage on the Bead Ruptor Elite. Load the screw capped tubes onto the 2 mL carriage, then tighten the fingerplate, and close the lid.
2. Homogenize at 4.2 m/s and room temperature for 30 s.
3. Some froth may be generated within the tubes because of the homogenization; this is normal. To alleviate any frothing in the tubes, remove them from the Bead Ruptor Elite and spin the tubes in a microcentrifuge up to 10,000 × *g* for no more than 15 s.

D. RT-qPCR

1. Prepare the RT-qPCR HCoV-229E N Primer Mastermix, as described in the recipes section.
2. Prepare the RT-qPCR mixture using the Luna Universal One-Step RT-qPCR kit, as described in the table below (Table 1).

Table 1. Luna Universal One-Step RT-qPCR mixture for RT-qPCR

Reagent	Volume per reaction
Luna Universal One-Step Reaction Mix (2×)	10 µL
Luna WarmStart® RT Enzyme Mix (20×)	1 µL
Forward Primer (5 µM)	1.25 µL
Reverse Primer (5 µM)	1.25 µL
Template RNA	1 µL

Nuclease-Free Water	5.5 µL

3. Prepare the PCR plate with identified spots for positive controls of purified HCoV-229E RNA, the unknown samples of homogenized HCoV-229E, and negative controls using DPEC and no HCoV-229E RNA.
4. Load 1 µL of sample (positive, unknown, or negative) into each well, as shown in the table above.
5. Seal the PCR plate with the plate sealing film.
6. Place plate into the CFX Connect and run the RT-qPCR with the following cycle parameters:
   a. 55°C for 30 min
   b. 95°C for 1 min
   c. 44 cycles of 95°C for 15 s and 56°C for 30 s
   d. Hold at 4°C for further processing

E. Amplicon Confirmation

1. Prepare a 2% agarose gel using TBE buffer and 5 µL of 10 mg/mL ethidium bromide stock for amplicon visualization. Both 15 well and 8 well combs can be utilized, to create wells large enough to accommodate the 20 µL volume of sample. Herein, we chose to use 15 well combs in our gel due to the large number of samples we were trying to visualize.
2. Load 20 µL of trypan blue into the 20 µL of suspected amplicon following RT-qPCR (1:1 mixture).
3. Load 20 µL of the amplicon/trypan mixture into each well of the 2% agarose gel, with 3 µL of 100 bp molecular ruler in 12 µL of trypan blue into one of the wells.
4. Run the gel at 125 V for approximately 1 h, or until the bands have sufficiently separated.
5. Visualize the gel utilizing the Gel Doc EZ Imager System, to observe the size of amplicon product in comparison to the ladder. The EZ Imager Software is set to standard ethidium bromide exposure settings with targeting of band intensities for imaging of the gels. This allows for identification of the target amplicon size for the N gene mixture at 308 bp.

Data analysis

In accordance with US CDC and World Health Organization (WHO) recommendations, detection of coronavirus associated disease should be deemed as positive in PCR detection for Ct values less than 40 (Bustin *et al.*, 2020 and Cheng *et al*., 2020). While these recommendations were put forward for SARS-CoV-2 detection, we have adopted them to serve as an appropriate cut off for positive detection of HCoV-229E in our protocol as well. When utilizing the $Ct < 40$ as the cut off for RT-qPCR, we still wanted to confirm the presence of an appropriate amplicon size (308 bp) via gel visualization, given the use of standard nucleotide primers that have the potential to dimerize giving a false positive. We were able to confidently confirm the presence of HCoV-229E in our samples, as detected by RT-qPCR.

The data supporting the *in vitro* limit of detection studies described herein is available in open access from the Virology Journal (Morehouse *et al.*, 2020). Additionally, the manuscript presenting the clinical validation of this method for SARS-CoV-2 detection is also available as open access through PLOS ONE (Morehouse *et al.*, 2021).

Notes

While this protocol was completed using traditional nucleotide primer sets targeting the N gene of HCoV-229E, a substitution of traditional primers for fluorescent probes can be made. If probes are utilized in place of primers, a change in the RT-qPCR kit will also be required, to one compatible with fluorescent probes. Additionally, if probes are used, there is no need for amplicon visualization on agarose gel following RT-qPCR, as there is minimal risk of primer self-binding or dimerization when utilizing a fluorescent quenching system.

Acknowledgments

The authors would like to thank Karl Jahn and Pete Tortorelli of Omni International Inc for the organizational and financial support of our work in developing this methodology. Additionally, we would like to thank Rachel Nash and Leah Proctor for their continued encouragement of our research efforts, with a particular acknowledgment for their sacrifices during the early days of the COVID-19 pandemic to support our work on this project.
The authors would like to acknowledge that this protocol has been adapted from previous work by Morehouse *et al*. (2020) entitled "A novel two-step, direct-to-PCR method for virus detection off swabs using human coronavirus 229E" and published in *Virology Journal* on 25 August 2020 (doi: 10.1186/s12985-020-01405-y).

Competing interests

ZPM, CMP, and RJN are named inventors on the patent describing this method held by Omni International, A PerkinElmer Company, but have no personal financial interests to disclose in the direct commercialization of this technology. CMP, GLR, and RJN are all full-time employees of Omni International Inc, A PerkinElmer Company, and GLR and RJN have no personal financial interests in the performance of the company. CMP is a shareholder of PerkinElmer, holding a personal financial interest in the performance of the company. ZPM is in a consulting relationship with Omni International, A PerkinElmer Company and holds no personal financial interests in the performance of the company. ZPM and RJN are also associated with Jeevan Biosciences LLC, a company which is not involved in the work described within this manuscript.

Ethics

No human subjects, patient samples, or animal subjects were utilized in the protocol described herein, thus no IUCAUC or IRB approval was required.

References

Elnifro, E. M., Ashshi, A. M., Cooper, R. J. and Klapper, P. E. (2000). Multiplex PCR: optimization and application in diagnostic virology. *Clin Microbiol Rev* 13(4): 559-570.

Bustin, S. A. and Nolan, T. (2020). RT-qPCR Testing of SARS-CoV-2: A Primer. *Int J Mol Sci* 21(8).

Morehouse, Z. P., Proctor, C. M., Ryan, G. L. and Nash, R. J. (2020). A novel two-step, direct-to-PCR method for virus detection off swabs using human coronavirus 229E. *Virol J* 17(1): 129.

Morehouse, Z. P., Samikwa, L., Proctor, C. M., Meleke, H., Kamdolozi, M., Ryan, G. L., Chaima, D., Ho, A., Nash, R. J. and Nyirenda, T. S. (2021). Validation of a direct-to-PCR COVID-19 detection protocol utilizing mechanical homogenization: A model for reducing resources needed for accurate testing. *PLoS One* 16(8): e0256316.

Cheng, M. P., Papenburg, J., Desjardins, M., Kanjilal, S., Quach, C., Libman, M., Dittrich, S. and Yansouni, C. P. (2020). Diagnostic Testing for Severe Acute Respiratory Syndrome-Related Coronavirus 2: A Narrative Review. *Ann Intern Med* 172(11): 726-734.