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Semi-nested RT-PCR enables sensitive and high-throughput detection of SARS-CoV-2 based on melting analysis

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ABSTRACT

Background: Asymptomatic transmission was found to be the Achilles' heel of the symptom-based screening strategy, necessitating the implementation of mass testing to efficiently contain the transmission of COVID-19 pandemic. However, the global shortage of molecular reagents and the low throughput of available realtime PCR facilities were major limiting factors.

Methods: A novel semi-nested and heptaplex (7-plex) RT-PCR assay with melting analysis for detection of SARS-CoV-2 RNA has been established for either individual testing or 96-sample pooled testing. The complex melting spectrum collected from the heptaplex RT-PCR amplicons was interpreted with the support of an artificial intelligence algorithm for the detection of SARS-CoV-2 RNA. The analytical and clinical performance of the semi-nested RT-PCR assay was evaluated using RNAs synthesized *in-vitro* and those isolated from nasopharyngeal samples.

Results: The LOD of the assay for individual testing was estimated to be 7.2 copies/reaction. Clinical performance evaluation indicated a sensitivity of 100% (95% CI: 97.83–100) and a specificity of 99.87% (95% CI: 99.55–99.98). More importantly, the assay supports a breakthrough sample pooling method, which makes possible parallel screening of up to 96 samples in one real-time PCR well without loss of sensitivity. As a result,

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up to 8,820 individual pre-amplified samples could be screened for SARS-CoV-2 within each 96-well plate of realtime PCR using the pooled testing procedure.
Conclusion: The novel semi-nested RT-PCR assay provides a solution for highly multiplex (7-plex) detection of SARS-CoV-2 and enables 96-sample pooled detection for increase of testing capacity.
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1. Introduction

The present Coronavirus Disease 2019 (COVID-19) has been spreading progressively worldwide with consecutive emergence of new SARS-CoV-2 variants that were highly contagious and vaccine resistant [1]. In addition to high transmission rate [2], a significant number of infected cases show mild or no symptom [3,4]. Since high viral loads have been reported in the upper respiratory specimens of asymptomatic cases [4–6], who are socially active throughout their infection, they contribute to the exponential spread of SARS-CoV-2 in the community, leading to catastrophic consequences [7–9].

Thanks to remarkable sensitivity and specificity, the "gold-standard" realtime RT-PCR has been popular technique for identification and isolation of COVID-19 infected individuals, including pre-symptomatic, mildly symptomatic, and asymptomatic cases [10–12], even prior to shedding of infectious virus [13]. The massive employment of molecular testing for COVID-19 has proved successful in some countries in response to the onslaught of the virus [10,14]. A small city in Italy, Vo Euganeo, is an highlighted example of how screening for COVID-19 based on molecular testing allowed for identification of asymptomatic cases, and eventually eliminated the virus in the population [15]. While early screening for SARS-CoV-2 is critical in containing the spread of the epidemic, it is equally important to prevent a second wave of infection in the later stages of the pandemic, when governments gradually start easing lockdown measures [8].

Since current molecular assays mostly employ 96-well real-time RT-PCR platform for detecting SARS-CoV-2 RNA [16], no more than 94 individual samples can be tested in about two hours with each platform. Providing exponentially increasing numbers of persons under investigation, limited number of real-time PCR instruments makes it impractical to implement effective mass testing programs [11]. In order to increase the testing throughput and minimize reagent consumption, a single-tube multiplex RT-PCR assay and pooled testing would be favorable. However, a possibly resulting attenuation in sensitivity would need to be carefully assessed [17,18]. On the other hand, the SARS-CoV-2 has continuously mutated over time, leading to growing number of primer/probe mismatches [19], which potentially impair current COVID-19 molecular assays due to the limited number of included target regions. As it is evidenced, an analysis of the GISAID database showed that a large number of published genome sequences harbor mutations at primer/probe binding regions of certain diagnostic assays, which can significantly affect their sensitivity (see Supplementary material 1*)*. In fact, the US FDA has recently updated lists of molecular tests that could be negatively impacted by viral mutations [20].

To address these pressing issues, we aimed to develop a novel semi-nested RT-PCR assay based on melting analysis to enable sensitive and high-throughput screening for SARS-CoV-2. The analytical and clinical performance of the assay have been evaluated for COVID-19 screening in the currently ongoing pandemic.

2. Material and methods

2.1. Primer design

Primers were designed for simultaneously targeting seven conserved genomic regions, including one fragment in the E protein gene, four fragments in the nucleocapsid (*N*) gene, and two fragments in the *ORF1ab* gene of SARS-CoV-2. All primer sequences are shown in Table 1. To avoid missing intended targets, exact match search was performed through all complete or near-complete genomes of SARS-CoV-2 isolates published in GISAID EpiCoV database (321,950 genomes, 21st Jan 2021) (https://www.gisaid.org/). Genomes with more than 1% of undetermined nucleotide (N) and/or with a length shorter than 20 kb were excluded from the search. The search was performed with perc_identity = 100 and blastn-short flag. The intended targets should be matched with the primers and in the right order/orientation and within expected distances.

The analysis scripts were implemented in Python 3.6 and are available on GitHub at https://github.com/leducquangpm/corona-primer-checker.

2.2. SARS-CoV-2 RNA samples

2.2.1. In-vitro transcribed RNA samples

Target sequences of SARS-CoV-2 genome were synthesized as dsDNA G-block (Phusa Biochem, Vietnam), and then *in-vitro* transcribed to generate RNA templates for development and validation of the assay. The first published SARS-CoV-2 sequence in GenBank (accession number MN908947) was used as the reference sequence. After purification, the copy number of *in-vitro* transcribed RNA was quantified by droplet digital PCR, and then mixed in equimolar concentrations to represent the wild-type genome of the SARS-CoV-2 (Positive control). Details of the transcription and quantification are shown in Supplementary material 2.

2.2.2. Simulated variants of SARS-CoV-2 RNA

The viral genomic variants were simulated by leaving out any one or more RNA target sequences from the wild-type mix of seven *in-vitro* transcribed RNA target sequences. In total, 126 simulated variants of SARS-CoV-2 RNA containing at least one out of seven *in-vitro* transcribed RNA target sequences were generated. Melting spectra resulted from the semi-nested RT-PCR using the RNA templates of either wildtype SARS-CoV-2 RNA, its simulated variants at varying concentrations and combinations (positive samples) or clinical samples negative with SARS-CoV-2 RNA were included for training the algorithm of melting data analysis.

2.2.3. Clinical samples

A total of 1,756 upper respiratory specimens, including 168 samples positive with SARS-CoV-2 RNA and 1,588 samples negative with SARS-CoV-2 RNA, were used for validation in this study. The Charité real-time RT-PCR assay [21] was performed as a reference method for detection of SARS-CoV-2 RNA in these clinical samples. The use of clinical samples was approved by the institutional review board and the ethics committee of the Vietnam National Hospital for Tropical Diseases. Total RNAs were extracted using either QIAmp Viral RNA kit (Qiagen, Germany), or MagNA Pure DNA and Viral NA Small Volume kit (Roche, Germany) according to manufacturer's instructions. All RNA samples were stored at −80 °C until use.

2.3. Machine learning for melting data analysis

We utilized gradient boosted trees for performing binary classification, i.e., predicting positive or negative outcomes. Gradient boosting is a powerful machine learning technique to devise strong predictive models by iteratively combining weaker ones [22].

For modeling, we utilized the melting spectrum between 70 °C and 95 °C (inclusive). We normalized each spectrum to a range of [0,1] by simple min–max scaling. Such normalization enabled us to be able to standardize melting spectra coming from varying settings or experiment batches. Then we calculated the change in this normalized melting spectrum along 0.2 °C steps, resulting in 126 features (attributes) for modeling. For optimizing the hyper-parameters of the gradient boosting classifier (maximum allowed decision tree depth, learning rate, regularization coefficient, and bagging temperature), we utilized Bayesian optimization with Gaussian Processes [23] in a 5-fold cross validation scheme. During hyper-parameter optimization, we tried to find the classifier parameters that maximize the mean F1-score calculated from the validation sets among 5 folds. F1-score is a well-established performance metric for binary classifiers and corresponds to the harmonic mean of precision and recall. A perfect model will achieve a score of 1.0 while a model that fails to predict the ground truth for each case will have 0.0 F1-score.

Our algorithms were implemented in the Python programming language (version 3.7) and training was performed on a 64-bit, 20-core machine in the Linux operating system. For gradient boosted tree classification, we used catboost library [24]. The machine-learning algorithm is available as a cloud-based application at https://htpcr.topdatascience.com/, allowing users to upload the melting data files produced by real-time PCR instruments, and receive testing results for the whole run of realtime PCR. Multiple analysis can be simultaneously processed with this cloud-based application (a trial account is available, with the username: editor, and password: tXPy5EzJFqmp).

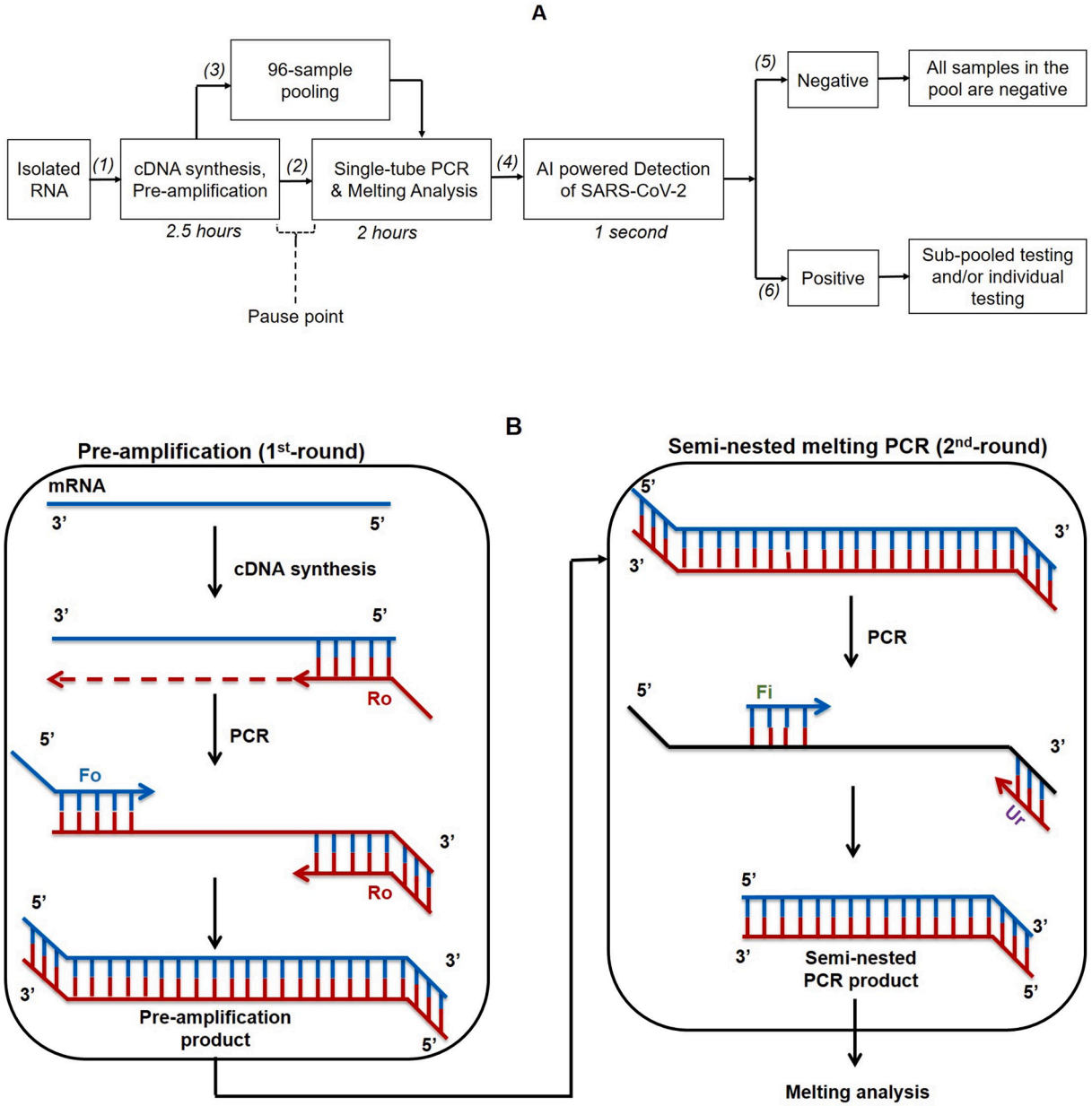


Fig. 1. Overview of the assay. A: (1) cDNA was synthesized from viral RNA and then pre-amplified using a conventional thermocycler (2.5 h); Pre-amplified cDNA samples were either (2) individually amplified or (3) pooled then amplified in a single-tube PCR reaction (1.5 h), prior to (4) Melting Analysis for the detection of SARS-CoV-2; (5) If a pool is reported as negative with SARS-CoV-2, all samples included in that pool are reported negative; (6) If a pool is positive with SARS-CoV-2, the pre-amplified cDNA samples included in the positive pool are sub-pool tested and/or individually tested following the plan created by the cloud-based application. B: Principles of the heptaplex semi-nested PCR, which includes the pre-amplification and melting semi-nested PCR reaction; Fo: outer forward primer, Ro: outer reverse primer, Fi: inner forward primer.

2.4. Assay procedure

The semi-nested RT-PCR assay includes two major steps: (1) pre-amplification and (2) semi-nested amplification and detection (Fig. 1). Briefly, RNA samples were individually reverse transcribed to cDNA and then enriched during the 1st step of pre-amplification. The pre-amplified products were further amplified in the 2nd step of semi-nested PCR amplification and then the raw melting profile data was analyzed by the cloud-based application for melting-based detection of SARS-CoV-2 RNA.

For individual testing

The pre-amplification step or 1st-round amplification was performed with a conventional thermocycler (Mastercycler® Pro S, Eppendorf, US). A 25 μL reaction was prepared containing 5 μL of RNA template; 10 μL of Pre-amplification master mix containing 2.5X HOT FIREPol® SolisGreen® qPCR Mix (Solis Biodyne), 0.5 units WarmStart® RTx Reverse Transcriptase (New England BioLabs), 1.25 M betaine solution (Sigma-Aldrich), 12.5% Glycerol (Thermo Scientific); 1.25 μL of Oligo P mix containing seven pairs of outer forward primers (Fo) and outer reverse primers (Ro), which were shown in Table 1A; and 8.75 μL of DEPC water. Thermal cycling program included an initial reverse transcription step at 50 °C for 10 min; then reducing temperature from 50 °C to 40 °C with 1 °C every minute; then 95 °C for 15 min for initial denaturation, followed by 6 cycles of 94 °C for 15 s, 60 °C for 3 min and 72 °C for 30 s; followed by 22 cycles of 94 °C for 15 s, 76 °C for 3 min, and 72 °C for 30 s; and a final extension at 72 °C for 5 min.

The 2nd-round amplification, performed with either a conventional thermocycler or with a realtime PCR platform, involves the 20-μL PCR reaction, which consists of 2 μL of the pre-amplification product (diluted 5 times by ultrapure water) as DNA template; 8 μL of Detection master mix containing 2.5X HOT FIREPol® SolisGreen® qPCR Mix (Solis Biodyne), 0.8 M Betaine solution (Sigma-Aldrich) and 3.75% DMSO (Solis Biodyne); 1 μL of Oligo D mix containing seven inner forward primers (Fi) and the single reverse primer (Ur) targeting the 5′-tail sequence that is universal for all of seven outer reverse primers (Table 1B); and 9 μL of ultrapure water. Thermal cycling was performed at 95 °C for 15 min for initial denaturation; followed by 45 cycles of 94 °C for 15 s, 63 °C for 30 s, and 72 °C for 30 s.

The melting profile was collected during a heating program from 70 °C to 95 °C with the increase of 0.2 °C every step, using either one of the following real-time PCR systems: Rotor-Gene Q 5plex HRM (QIAGEN, Germany), LightCycler® 480 system (Roche), CFX96 Touch Real-Time PCR Detection System (Bio-Rad), ABI 7500 Fast & 7500 Real-Time PCR system (Thermo Fisher), and QuantStudio 3/5 Real-Time PCR System (Thermo Fisher). The raw melting profile data was exported to an excel file, then uploaded to the cloud-based application for melting-based detection of SARS-CoV-2.

For pooled testing

The semi-nested RT-PCR assay supports pooling of up to 96 samples after pre-amplification step. For each testing batch of up to 8,820 samples, the pooling instruction and testing plan is automatically generated by the cloud-based application at https://htpcr.topdatascience.com/. In brief, RNA samples were individually processed with the pre-amplification step as individual testing. An aliquot of pre-amplification product derived from each of up to 96 samples on an 8x12 plate were pooled together, then the pooled samples were diluted 5 times to be used as template for the 2nd-round amplification and melting-based detection step. PCR mix preparation, thermal cycling and melting analysis were performed as individual testing. If a pool is negative with SARS-CoV-2, all samples in that pool will be reported as negative by the cloud-based application. If a pool is positive with SARS-CoV-2, the cloud-based application will automatically generate the plan for sub-pool or individual testing (2nd- round amplification) with the pre-amplified products corresponding to that pool as template, to find out which sample is positive or negative. The underlying principle for the cloud-based application to generate the pooling instruction and

Table 1A
Primers used in the Oligo P mix, consisting of outer forward primers (Fo) and outer reverse primers (Ro).

List	Primer name	Target gene/ Nucleotide position*	Sequence (5′- 3′)	Conc** (μM)
1	Fo-E	E/26203	CGACGTAAAACGACGGCCAGT**ACTAGCG TGCCTTTGTAA**	0.8
2	Ro-E	E/26395	CACACAGGAAACAGCTATGACCATG**GAC TCACGTTAA**	3.2
3	Fo-ORF1ab-1	ORF1ab/13262	CGACGTAAAACGACGGCCAGT**CACATAG ATCATCCAAATCCT**	0.8
4	Ro- ORF1ab-1	ORF1ab/13506	CACACAGGAAACAGCTATGACCATGA**GC ACGGTGTAAGA**	3.2
5	Fo- ORF1ab-2	ORF1ab/15358	CGACGTAAAACGACGGCCAGTT**CGCAAA CATACAACGT**	0.8
6	Ro- ORF1ab-2	ORF1ab/15544	CACACAGGAAACAGCTATGACCATG**CCG TGACAGCTT**	3.2
7	Fo-N1	N/28193	CGACGTAAAACGACGGCCAGTA**GCGTTG TTCGTTCTATGA**	0.8
8	Ro-N1	N/28397	CACACAGGAAACAGCTATGACGATG**TG CCGACGTTGT**	3.2
9	Fo-N2	N/28593	CGACGTAAAACGACGGCCAGT**GATGGT ATTTCTACTACCTAGGAA**	0.8
10	Ro-N2	N/28840	CACACAGGAAACAGCTATGACCATG**AC GTGATGAGGAA**	3.2
11	Fo-N3	N/28869	CGACGTAAAACGACGGCCAGT**CAGGCA GCAGTAGGGGAAC**	0.8
12	Ro-N3	N/29000	CACACAGGAAACAGCTATGACCATG**CTT GTTGTTGTTG**	3.2
13	Fo-N4	N/29064	CGACGTAAAACGACGGCCAGT**CCACTA AAGCATACAAT**	0.8
14	Ro-N4	N/29267	CACACAGGAAACAGCTATGACCATG**TC AACCACGTTC**	3.2

* : Start position at the 5′-end of the priming sequence.
** Conc: Concentration of individual primer in the primer mix.

Table 1B
Primers used in the Oligo D mix, consisting of inner forward primers (Fi) and universal reverse primer (Ur).

List	Primer name	Target gene/ Nucleotide position*	Sequence (5′- 3′)	Conc** (μM)
1	Fi-E	E/26269	ACAGGTACGTTAATAGTTAATAGCGT	6
2	Fi- ORF1ab-1	ORF1ab/13342	CCCTGTGGGTTTTACACTTAA	8
3	Fi- ORF1ab-2	ORF1ab/15431	GTGAAATGGTCATGTGTGGCGG	12
4	Fi-N1	N/28287	GACCCCAAAATCAGCGAAAT	8
5	Fi-N2	N/28681	GGGAGCCTTGAATACACCAAAA	8
6	Fi-N3	N/28881	GGGGAACTTCTCCTGCTAGAAT	4
7	Fi-N4	N/29164	TTACAAACATTGGCCGCAAA	8
8	Ur		CACACAGGAAACAGCTATGACCATG	8

*: Start position at the 5′-end of the priming sequence.
** Conc: Concentration of individual primer in the primer mix.

testing plans is shown in the Supplementary material 3. Further information could be found in the cloud-based application using the trial account.

2.5. Analytical performance

2.5.1. Analytical sensitivity

Limit of detection (LOD) of the assay in individual testing was determined using known concentrations of generated *in-vitro* SARS-CoV-2 RNA spiked into extracted negative clinical samples. Each concentration was tested in various replicates (Table 2), and the LOD was defined as the amount of SARS-CoV-2 RNA that could be detected in 95% of replicates.

LOD of the assay in 96-sample pooled testing was also determined. Positive samples were created by spiking *in vitro* RNA into extracted negative clinical samples, using the same concentrations as individual testing. The pre-amplification product of a positive sample was mixed with pre-amplification products of 95 negative samples to make the positive pooled template for the 2nd amplification. LOD of the pool testing was determined by the same method as the individual testing.

2.5.2. Analytical specificity

For in silico cross-reactivity check, the NCBI's BLASTN suite was used to search each of the primers against the BLAST Nucleotide collection database and Betacoronavirus nucleotide sequence database to avoid amplification of unintended targets. The search was performed with perc_indentity = 80 and blastn-short flag. Sequences matched the primers with at least 80% homology in the right order/orientation and within expected distances were considered as potential agents causing false positives. The analysis scripts were implemented in Python 3.6 and are available on GitHub at https://github.com/leducquangpm/corona-primer-checker.

Seven viruses that are closely related to SARS-CoV-2 and 16 additional organisms were empirically tested for the specificity of the assay. All samples were stored at −80 °C until use. Each organism was tested twice. List of the organisms and test results are shown in Table 3.

Table 2
LOD study of the semi-nested RT-PCR assay.

SARS-CoV-2 RNA concentrations (copies/reaction)	Number of Replicates	Positive rates (individual testing)	Positive rates (pooled testing)
5	25	23/25 (92%)	24/25 (96%)
10	25	24/25 (96%)	24/25 (96%)
20	25	25/25 (100%)	25/25 (100%)
50	25	25/25 (100%)	25/25 (100%)
100	25	25/25 (100%)	25/25 (100%)
500	6	6/6 (100%)	6/6 (100%)
2,500	6	6/6 (100%)	6/6 (100%)
5,000	6	6/6 (100%)	6/6 (100%)
Total	143		–

Table 3
Organisms tested for assay specificity.

Organism	Material	Test concentration (copy/ml)	Semi-nested RT-PCR result
Human coronavirus 229E	Twist Synthetic RNA	5×10^5	Not detected
Human coronavirus NL63	Twist Synthetic RNA	5×10^5	Not detected
Human coronavirus OC43	Twist Synthetic RNA	5×10^5	Not detected
MERS-coronavirus	Twist Synthetic RNA	5×10^5	Not detected
SARS-coronavirus	Twist Synthetic RNA	5×10^5	Not detected
Bat coronavirus	RNA extracted from bat's liver tissue (NIHE)	unknown	Not detected
Porcine epidemic diarrhea virus	Culture (VNUA)	2×10^6	Not detected
Human enterovirus 68	Twist Synthetic RNA	5×10^5	Not detected
Human bocavirus 1	Twist Synthetic RNA	5×10^5	Not detected
Human parainfluenza virus 1	Twist Synthetic RNA	5×10^5	Not detected
Human parainfluenza virus 2	EQA inactivated virus (OUCRU)	2×10^6	Not detected
Human parainfluenza virus 3	EQA inactivated virus (OUCRU)	2×10^6	Not detected
Human parainfluenza virus 4	Twist Synthetic RNA	5×10^5	Not detected
Human rhinovirus 89	Twist Synthetic RNA	5×10^5	Not detected
Influenza A virus H1N1	Twist Synthetic RNA	5×10^5	Not detected
Influenza B	Twist Synthetic RNA	5×10^5	Not detected
Influenza H3N2	Twist Synthetic RNA	5×10^5	Not detected
Influenza H5N1	EQA inactivated virus (OUCRU)	2×10^6	Not detected
Influenza H7N9	EQA inactivated virus (OUCRU)	2×10^6	Not detected
Influenza H5N6	EQA inactivated virus (OUCRU)	2×10^6	Not detected
Influenza A untyped	EQA inactivated virus (OUCRU)	2×10^6	Not detected
Measles	Twist Synthetic RNA	5×10^5	Not detected
Mumps	Twist Synthetic RNA	5×10^5	Not detected

*NIHE: National Institute of Hygiene and Epidemiology; OUCRU: Oxford University Clinical Research Unit (Hanoi, Vietnam); VNUA: Vietnam National University of Agriculture (Hanoi, Vietnam); EQA: External Quality Assessment.

2.6. Clinical performance

For individual testing, clinical performance of the semi-nested RT-PCR assay was evaluated using 1,756 archived RNA samples including 168 confirmed positive samples and 1,588 confirmed negative samples with the reference assay [21].

For pooled testing, positive pools were prepared by combining pre-amplification products of one positive sample and those of 95 random negative samples. Negative pools were created by combining pre-amplification products of 96 random negative samples. In total, 157 positive pools and 136 negative pools were tested. In addition, 96-sample pools containing more than one positive clinical sample (2, 5, 10, 20, or 50) were generated in 10 replicates and then tested with the assay.

2.7. Statistical analysis

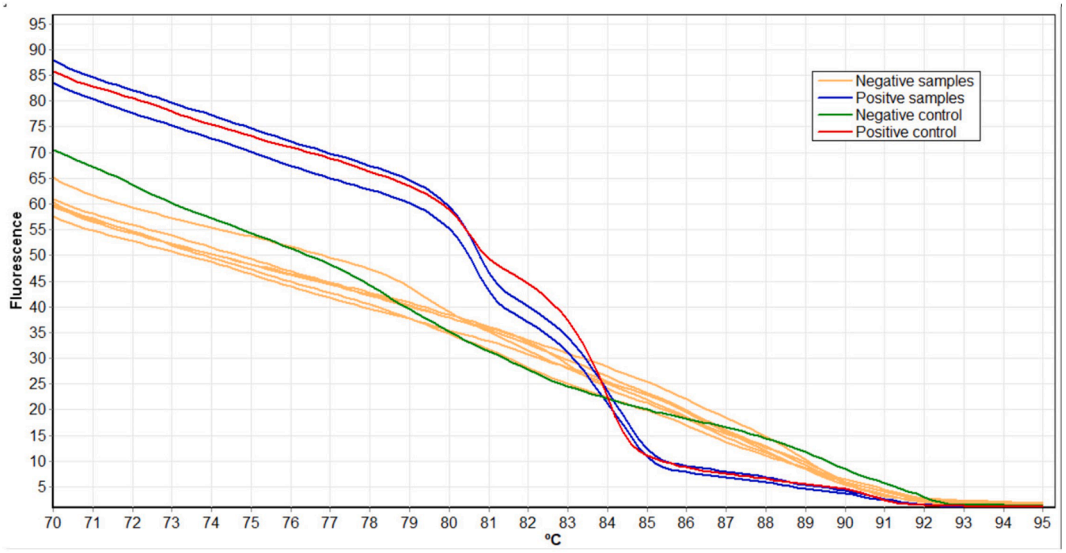
LOD of the assay was estimated by PODLOD calculation program version 9 with 95% confidence intervals (95% CI). The degree of agreement between the assay and the Charité assay recommended by WHO was assessed using the Cohen's Kappa coefficient. Sensitivity and specificity were analyzed by MedCalc (Version 19.2.0, MedCalc Software Ltd, Ostend, Belgium).

3. Results

3.1. Overview of the assay

The semi-nested RT-PCR assay has been developed for simultaneous amplification of seven distinct target sequences specific for SARS-CoV-2 in a single tube. The workflow of the assay is outlined in Fig. 1A. Seven regions are concurrently amplified in order to minimize the false negatives arising from low viral load samples [25] and/or viral mutations [26]. For each target sequence, a semi-nested RT-PCR was designed with the outer reverse primer (Ro) containing a universal 5′-tail sequence (Fig. 1B: Pre-amplification). Consequently, a single reverse primer corresponding to this universal 5′-tail sequence (Universal reverse primer) is able to amplify all seven target sequences in the presence of appropriate inner forward primers (Fi) during the 2nd round of semi-nested amplification (Fig. 1B). Such a design lessens the number of primers employed in the multiplex PCR reaction tube, thus reducing the nonspecific amplification, and improving the specificity of the melting-based PCR. The melting spectrum generated from the assay is a complicated sum of seven individual overlapping melting spectra. Examples of melting data output are shown in Supplementary material 4. An artificial intelligence model, integrated in the cloud-based

A



B

Well Position	Pool/Sample Position	Sample Name	Result
1	S01-A-1	1: Negative sample	Negative
2	S01-B-1	2: Negative sample	Negative
3	S01-C-1	3: Negative sample	Negative
4	S01-D-1	4: Negative sample	Negative
5	S01-E-1	5: Negative sample	Negative
6	S01-F-1	6: Negative sample	Negative
7	S01-G-1	7: Positive sample	Positive
8	S01-H-1	8: Positive sample	Positive

Fig. 2. Detection of SARS-CoV-2 RNA with the semi-nested RT-PCR assay. (A) Representative raw melting spectra resulted from 2 samples positive with SARS-CoV-2 RNA (blue lines: Positive samples), 6 samples negative with SARS-CoV-2 RNA (orange lines: Negative samples), positive control (red line) and negative control (green line); and (B) Output generated with the cloud-based application that interprets the melting spectra collected from each well of the realtime PCR instrument.

application, was specifically designed to automatically discriminate the raw melting spectra of positive samples from those of negative samples (Fig. 2).

When the 96-sample pooled testing procedure is used, the cloud-based application will automatically create the testing plan, sub-pool plan, and finally identify which individual samples positive or negative with SARS-CoV-2 RNA based on the melting data. Up to 8,820 samples could be simultaneously proceeded within one batch of pooled testing that involves only one run of 96-well realtime PCR in the 2^n-round amplification step.

3.2. Limit of detection (LOD)

In individual testing, results showed that 92% of the replicates tested positive with 5 copies/reaction, and 96% of samples containing at least 10 copies/reaction tested positive (Table 2). The LOD was estimated to be 7.2 copies/reaction (95% CI: 4.7 – 10.9 copies/reaction). Using the same standard samples of SARS-CoV-2 RNA, the LOD of the Charité's assay recommended by WHO was 50 copies/reaction, which is 6.9 times higher than that of the semi-nested RT-PCR assay.

The LOD of the assay in pooled testing was estimated to be 6.4 copies/reaction (95% CI: 4.1–10.0 copies/reaction). Detail results are shown in Table 2.

3.3. Cross-reactivity with other respiratory viruses or other coronaviruses

With the exception of betacoronaviruses, the in-silico analysis showed that there was no microorganism, found with ≥ 80% homology to primer sets of the semi-nested RT-PCR assay. In the betacoronavirus group, there are 346 sequences/genomes found with ≥ 80% homology to designed primers. Those sequences originated from Pangolin coronavirus, Bat SARS-like coronavirus, bat coronavirus and SARS-CoV-1 isolates.

Among the highly similar genomes, the Pangolin coronavirus, bat SARS-like coronavirus and bat coronavirus are not direct human infectious organisms while SARS-CoV-1 caused human severe acute respiratory pandemic in 2003. Thus, SARS-CoV-1 was empirically tested with the assay. Besides, other available coronaviruses, and sixteen additional organisms were also experimentally tested with the assay. None of the assays were cross-reactive to any of the tested organisms. Detailed results are shown in Table 3.

Comparative clinical performance of the semi-nested RT-PCR assay versus WHO recommended Charité assay

Detection of SARS-CoV-2 by the semi-nested RT-PCR assay and the Charité assay [21] in 1,756 specimens is shown in Table 4. The novel assay successfully identified 168/168 positive samples and 1,586/1588 negative samples, corresponding to a sensitivity of 100% (95% CI: 97.83% – 100%), and a specificity of 99.87% (95% CI: 99.55% – 99.98%). The Cohen's Kappa coefficient of 0.9935 reveals the almost perfect agreement between two assays in detecting SARS-CoV-2 RNA.

Table 4
Comparison between the semi-nested RT-PCR and the Charité assays in detection of SARS-CoV-2 in clinical samples.

Semi-nested RT-PCR	Charité assay	
	Positive	Negative
Positive	168	2
Negative	0	1586
Sensitivity (%)	100% (95% CI: 97.83% – 100.00%)	
Specificity (%)	99.87% (95% CI: 99.55% – 99.98%)	
Cohen's Kappa coefficient	0.9935	

3.5. Clinical performance of the semi-nested RT-PCR assay in pooled testing

Regarding the 96-sample pools that involved a single positive sample and 95 negative samples, 155/157 positive pools were detected as positive and 136/136 negative pools were detected as negative. The results showed a sensitivity of 98.73% (95% CI: 95.47% to 99.85%) and a specificity of 100% (95% CI: 97.32% to 100%). In addition, SARS-CoV-2 RNA was successfully detected in 100% (50/50) of 96-sample pools with multiple positive samples (2, 5, 10, 20, or 50) using the semi-nested RT-PCR assay.

4. Discussion

Despite remarkable sensitivity and specificity, the high cost and limited throughput of current gold-standard assays based on real-time RT-PCR technology is not sufficient for massive testing-based screening for COVID-19, especially in low- and middle-income countries. In this study, we have developed and validated a novel semi-nested RT-PCR assay, as a potential candidate for massive screening for COVID-19. The assay employs end-point detection based on multi-amplicon melting analysis in a single tube with the support of an advanced machine-learning algorithm. The semi-nested RT-PCR assay makes possible 96-sample pooled testing that is able to screen for thousands of samples in one real-time PCR run.

Our data showed that the clinical performance of the semi-nested RT-PCR was comparable to the WHO recommended Charité assay [21] with 100% sensitivity and 99.87% specificity in individual testing. The assay had a LOD of 7.2 copies/per reaction, which is about seven times lower than that of the Charité assay when the same RNA standards were used. The assay successfully discriminated SARS-CoV-2 from seven closely related coronaviruses, and other respiratory viruses that can cause similar symptoms to COVID-19. Altogether, these data show that the performance of the assay is suitable for use as a screening test.

As opposed to most currently available COVID-19 real-time RT-PCR assays, wherein only two or three regions of the viral genome are targeted at maximum, the semi-nested RT-PCR assay simultaneously amplifies seven genomic regions of SARS-CoV-2 in a single tube. This is particularly important in response to the emergence of numerous SARS-CoV-2 variants with new mutations [19,26,27] that may cause false negatives due to mismatches at binding sites of the primers/probes on target sequences. Several studies have demonstrated that mutations in new variants such as Delta and Omicron significantly affected the sensitivity of certain realtime RT-PCR test kits and their detection limit [28–30]. Even though nucleotide substitutions are already prevalent (see Supplementary material 1), mutations would unlikely happen concurrently in all seven regions targeted by this semi-nested RT-PCR assay. New emerging variants could still be identified using the novel assay based on the remaining un-mutated targets. Based on SARS-CoV-2 sequences published on the GISAID database, updated 21st Jan 2021 (Supplementary material 5), our analysis showed that there are only 2 out of 321,950 (0.0006%) high-quality genomes harbor mutations at 6 and 7 target regions. More recently, the SARS-CoV-2 variant of Omicron was shown to contain mutations in only three out of seven regions targeted by the semi-nested RT-PCR. Since the simulated variants with up to six target sequences missing could still be identified as positive, it is unlikely that the semi-nested RT-PCR assay fails to detect SARS-CoV-2 RNA with currently published sequences.

In contrast to existing real-time RT-PCR assays that collect fluorescent signals generated from the Taqman probes at every cycle during amplification reaction, the semi-nested RT-PCR collects melting data at the end of the SYBR green-based PCR for the detection of SARS-CoV-2. It is also worthy noted that the pre-amplification step and the 2nd-round amplification can be performed using a conventional thermocycler, before being subjected to a real-time PCR instrument for melting-based analysis in just about 15–30 min. This significantly decreases the

demand for real-time PCR instruments that are dozen times more expensive than conventional thermocyclers. In addition, the assay avoids the use of fluorescence-labeled probes, thus minimizing reagent costs and greatly accelerating production of test kits for global use.

To maximize the throughput of testing and optimize the testing cost, sample pooling has long been adopted when screening for an infectious agent at a low disease prevalence in a large population [31]. However, the assay sensitivity can be significantly attenuated with traditional pooling methods [32]. The higher the number of samples being tested in a pool, the lower concentration of targeted analytes in the pooled samples and the lower likelihood of being detected as positive individuals. By contrast, the novel pooling strategy utilized in this study has no impact on assay sensitivity as it increases the copy number of target sequences in the pre-amplification step prior to pooling. Our data showed that the 96-sample pooled testing detected SARS-CoV-2 RNA with a detection limit comparable to that of individual testing. At a certain stage of an outbreak, the prevalence of SARS-CoV-2 infected cases can be extremely low in certain populations or sub-populations, with only a few cases among hundreds or even more of tested subjects. 96-sample pooling with this novel assay allows for 96 individual samples to be screened for SARS-CoV-2 within one well of real-time PCR in few hours. As a result, up to 8,820 samples could be individually pre-amplified in parallel using sufficient number of conventional thermocyclers, followed by 96-sample pooling to generate up to 92 pools as templates for the 2nd round of amplification, which could be accomplished using just one platform of 96-well realtime PCR.

5. Conclusions

In conclusion, with the tolerability to viral mutations and the capacity of pooling of up to 96 pre-amplified samples, the semi-nested RT-PCR assay is a sensitive and high-throughput screening assay for COVID-19. Thanks to the pre-amplification step, the assay could detect SARS-CoV-2 RNA in individual clinical samples as well as 96-sample pools with a low limit of detection. Given a significantly reduced demand for real-time PCR instruments and expensive fluorescence-labelled reagents, the semi-nested RT-PCR assay holds great promise to be implemented for the purpose of massive screening for SARS-CoV-2 and inspires further development of new screening assays for other infectious diseases and cancers at scale.

CRediT authorship contribution statement

Anh Thi Ngoc Nguyen: Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing, Writing – original draft. **Hoai Thi Bui:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing. **Quynh Thi-Huong Pham:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing. **Ly Thi Thao Hoang:** Methodology, Validation. **Hung Xuan Ta:** Data curation, Software. **Timo Heikkinen:** Data curation, Software. **Duyet Van Le:** Methodology, Validation. **Trang Dinh Van:** Methodology, Validation. **Nam Quoc Ngo:** Methodology, Validation. **Phuong Thi Hong Huynh:** Methodology, Validation. **Trang Thi Huyen Tran:** Methodology, Validation. **Hoan Quoc Phan:** Methodology, Validation. **Luong Van Hoang:** Methodology, Validation. **H.Rogier van Doorn:** Methodology, Validation. **Diep Thi Ngoc Nguyen:** Methodology, Validation. **Tam Thi Nguyen:** Methodology, Validation. **Nam Sy Vo:** Data curation, Formal analysis. **Cuong Viet Vo:** Methodology, Validation. **Sau Khac Trinh:** Methodology, Validation. **Tai The Pham:** Methodology, Validation. **Quang Duc Le:** Data curation, Formal analysis. **Phan Van Le:** Methodology, Validation. **Son Thai Nguyen:** Methodology, Validation. **Loan Thi Tran:** Data curation, Formal analysis. **Toan Dinh Vu:** Methodology, Validation. **Quynh Anh Nguyen Vu:** Methodology, Validation. **Nguyet Thi Trieu:** Methodology, Validation. **Thuy Thi Le:** Methodology, Validation. **Ung Dinh Nguyen:** Methodology, Validation. **Jakob Steman:** Conceptualization. **Tho Huu Ho:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – review & editing, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Competing interests

Authors state no conflict of interest.

The corresponding author of current manuscript, Tho Huu Ho, is also the inventor of the pending patent application (PCT/IB2021/052120), which described the novel method of pooling after the pre-amplification step for high-throughput molecular testing.

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Informed consent

Not applicable.

Ethical approval

The use of clinical samples was approved by the institutional review board and the ethics committee of the Vietnam National Hospital for Tropical Diseases.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2022.04.997.

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