

Detection of SARS-CoV-2 using high-throughput PCR

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ABSTRACT

Efficient management of an epidemic requires identification and isolation of infected individuals from healthy population to curb community spread of the pathogen. Therefore, a faster and efficient diagnostic method for population screening is desirable to flatten the curve of daily rise of cases. This will also help medical professional to treat the epidemic efficiently. The COVID 19 pandemic caused by SARS-CoV-2 coronavirus poses a huge challenge to human being and the countries with high-density population are facing unique challenges. For the SARS-CoV-2 pandemic, Quantitative Real Time Polymerase Chain Reaction (qRT-PCR) emerging as a gold standard for the identification of infection; however, infrastructure requirement for this assay is at a high-demand and therefore, resources are not abundant. We, therefore, propose a Polymerase Chain Reaction (PCR)-based sensitive method for the detection of SARS-CoV-2 coronavirus. The pipeline discussed in the paper will broaden the scope of control and surveillance of COVID 19 in a high-throughput manner without a sophisticated diagnostic infrastructure.

1. INTRODUCTION

A severe viral pneumonia outbreak at China was reported on December 2019 and the causative agent was identified as a novel coronavirus named as “SARS-CoV-2” [1]. Over the past few months, this newly discovered coronavirus has spread globally and caused more than 50,000 deaths. The coronavirus disease (commonly known as COVID-19) being highly contagious has rapidly spread from human to human across the world, through droplet infection. Over last 3 months, more than one million people are infected by the virus and a number of cases are growing alarmingly. Last month, this disease has been declared as a pandemic by the World Health Organization (WHO). With a rapid increase of demand of health-care facilities, COVID 19 pandemic is straining health care systems worldwide [2]. Today, even the developed countries, with excellent health care systems, are overstretched and unable to operate effectively due to the unprecedented inflow of patients every day. The disease poses a significant threat to the counties with comparatively weaker healthcare infrastructure. Considering population density of India (approximately 400/km²), the existing health-care facilities are certainly inadequate

to fight this pandemic. Therefore, preventive measures, such as social distancing, isolation of infected individuals, and nationwide lockdown are the sensible options that are being pursued in India to prevent community level transmission of COVID 19.

Globally, this extraordinary situation demands tailored solutions for COVID 19 diagnosis, immunization, and treatment. Considering the communicable nature of the disease, it is a prerequisite to develop an efficient and robust screening methodology to curb the spread of the virus [3]. Currently, the number of tests conducted per millions of people for mass screening of COVID 19 patients in India is comparatively less than the other countries, such as Singapore, South Korea, and Hong Kong. Various companies have come up with Quantitative Real Time Polymerase Chain Reaction (qRT-PCR), Reverse Transcription Loop Mediated Isothermal Amplification (RT-LAMP) (loop-mediated isothermal amplification), and antibody-based diagnosis kits for the identification of SARS-CoV-2 in suspected cases. The qRT-PCR and RT-LAMPse methods are costly as the process requires expensive instruments. On the other hand, the antibody-based diagnostics is yet to prove its precision during early days of infection.

Polymerase Chain Reaction (PCR) machines are more abundant than qRT-PCR machines in various universities, diagnostic testing centers, and colleges. The need of the hour is to utilize the available resources for expanding the diagnostics capacity and throughput. Therefore, the development of a PCR-based

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detection system for SARS-Cov-2 has tremendous potential to improve throughput of the sample screening in small towns and villages of India. Presently, only people with overt symptoms are getting tested. Unfortunately, most cases of COVID 19 are mild or asymptomatic, especially in the younger population, in early days of infection. Therefore, efficient mass-screening strategies should be deployed to estimate the spread of infection and implementation of quarantine plans to prevent community spread.

1.1. High-Throughput PCR-Based Detection

In the current report, we are highlighting a PCR-based COVID 19 detection strategy. Such tests use an approach to identify presence or absence of genetic material(s) of the virus in the samples. This PCR technique amplifies small stretch of viral genome using custom-designed “primers” at a high stringency condition. (a) The sample is collected from the oral/nasal cavity of suspect patients using a synthetic fiber swab with plastic shafts; (b) transit the swabs from the collection point to the testing point should be done in tubes containing viral transport media; (c) high-throughput RNA extraction from the swab samples; (d) cDNA synthesis of the extracted RNA in 96-well plate format; (e) 96-well plate PCR using selective primers using 10 picomoles of each forward and reverse primers; (f) capillary electrophoresis of the 96-well PCR plate to identify PCR products in positive samples. Primer pairs for three of more than three genes may be used in parallel amplification for statistical validation. Figure 1 depicts a schematic representation of the overall procedure for COVID 19 detection using PCR technology.

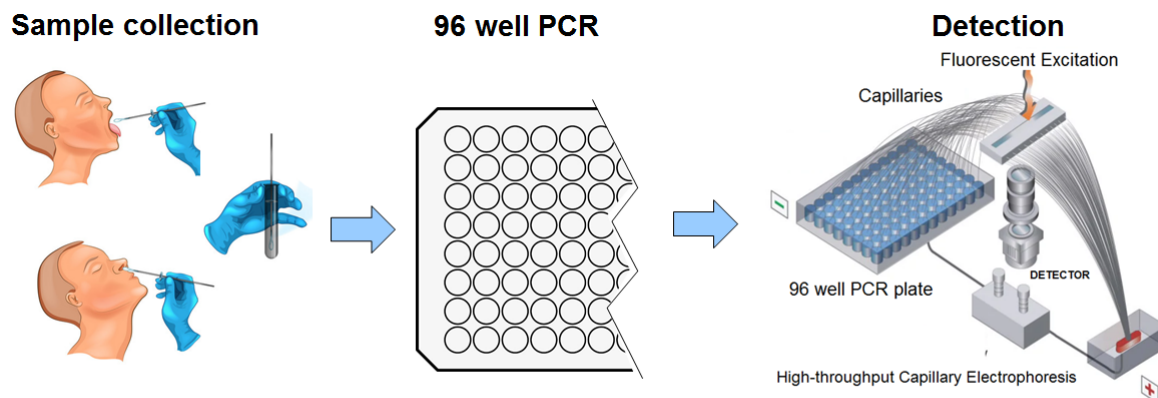
1.2. Reagents and Kits

As explained in Figure 1, the first step is the collection of samples on swab from the throat or nasal cavity in viral transport media (TMMEDIA, Delhi, India). The kit has one sterile flocked collection swab and a tube of Viral Transport from the place of

collection to testing laboratory. Buffers and antibiotics content of the medium are recommended by Centers for Disease Control and Prevention (CDC) and WHO to ensure the viability of the coronavirus during transport. The next step is the extraction of RNA from viral transport media in a 96-well plate format. High-throughput RNA extraction kits, such as PureLink™ Pro 96 total RNA Purification Kit (Thermo Fischer), E-Z 96® Total RNA Kit (Omega Bio-Tek), Mag-Bind® Total RNA 96 Kit (Omega Bio-Tek), and 96-well purification of total RNA from cells (Qiagen) could be used for this purpose. The third step is RT-PCR of the extracted RNA in a 96-well format using SuperScript™ III First-Strand Synthesis System (ThermoFischer) or TurboCapture 96 mRNA kit (Qiagen). The fourth step is 96-well PCR using the designed RIL-CoVID primers (Table 1) and 2× QIAGEN Multiplex PCR Master Mix (Qiagen) or AmplyMAX™ Multiplex PCR Master (Canvaxbiotech). PCR machines with 96-well thermal block of ThermoFischer, Biorad, or any other model could be used for PCR amplification. The detection of desired PCR products in 96-well plate could be done using 7100 CE System (Agilent) or Capillary Electrophoresis (SCIEX) as detailed in Figure 1.

1.3. Designing of Primers for SARS-CoV-2

Genome of 41 variants of SARS-CoV-2 was downloaded from NCBI and aligned using multiple sequence alignment (MSA) tool for phylogenetic analysis using MEGA 7.0 software package [4]. The phylogenetic tree constructed using 28.5 KB consensus DNA region indicated a high degree of similarities among the genomes of the various SARS-CoV-2 sequences at different part of the world (Fig. 2). We have identified some conserved regions from MSA alignment file and have designed PCR primers using an *in-house* algorithm. A set of 100 pairs of oligos were then subjected to similarity searches against other viral genomes and human genome. Finally, the primers which showed less than



Step 1: Extraction of RNA from Swab, and cDNA preparation in a high-throughput format

Step 2: identification of specific PCR amplification of the specific cDNA (50 – 100 nucleotide region)

Step 3: Capillary electrophoresis and detection of amplicon specific to SARS-Cov-2

Figure 1: Schematic diagram showing a process flow for PCR-based early detection of SARS-Cov-2 in throat/ nasal swab samples.

Table 1: List of RIL-COVID primers and probes for designing PCR or FISH based approach for detection of virus.

S.No	Primer Sequence	Primer Length	PCR product	Target gene Name	Gene details
1	Probe: ACAGTTTATGATCCTTTGCAAC	22	-	Surface glycoprotein	GenBank ID: QIK50448.1 Coordinates: 21563-25384 Gene Length: 3821
2	PF: TCGGAAGAGACAGGTACGTT	20	124	Envelope protein	GenBank ID: QIO04369.1 Coordinates: 26245-26472 Gene Length: 227 bp
3	RP: CAATATTGCAGCAGTACGCA	20	-		
4	Probe: CATCCTTACTGCGCTTCGAT	20	-	Membrane glycoprotein 'M'	GenBank ID: QIO04370.1 Coordinates: 26523-27191 Gene Length: 668 bp
5	PF: CTGACCAGACCGCTTCTAG	19	146		
6	RP: GAAAGCGTTCGTGATGTAGC	20	-		
7	Probe: GACCTGCCTAAAGAAATCACT	21	-		
8	PF: CAAGCTTTCGGCAGACGTG	19	155	Nucleocapsid phosphoprotein	GenBank ID: QIO04374.1 Coordinates: 28274-29533 Gene Length: 1259 bp
9	RP: TCCATGCCAATGCGCGAC	18	-		
10	Probe: GCTTCAGCGTTCTTCGGAA	19	-		
11	FP: TAATGGACCCAAAATCAGC	20	89		
12	RP: TCGTTCCTCCATTCTGGTTA	20	87		
13	FP: GAACTGATTACAAACATTGG	20	92		
14	RP: CTTCCATGCCAATGCGCGACA	21	-		
15	FP: TGCAACTGAGGGAGCCTTGA	20	-		
16	RP: CTTGAGGAAGTTGTAGCACG	20	-		

50% homology in human genome and 100% similarity with the genome of SARS-CoV-2 were analyzed further. Finally, the selected primers and probes were also subjected to similarity searches with the genome of other respiratory viruses available in non-redundant database. The unique primers which selectively binds to the genome of SARS-Cov-2 were selected. Table 1 has the list of primers and probes which can be used for PCR amplification and hybridization. The DNA fragments amplified using the PCR primers were identified as genes (partial) for surface glycoproteins, envelope protein “E”, membrane glycoprotein “M”, ORF3a protein and nucleocapsid phosphoprotein “S” (Table 1). Except ORF3a, all these proteins fall in the category of structural proteins which plays an important role in the assembly of virion capsid.

1.4. Community Infection Assay

The PCR method discussed here could also be used for scoring stage-3 of epidemic or community screening of infection by SARS-CoV-2. Pooled cDNA (mix of 10–12 samples) could be added in each well of PCR plate. After PCR amplification capillary electrophoresis of each well could be done to identify the well containing infected samples. During stage 1 and stage 2 of an epidemic, majority of samples tested (95%) are negative which results loss of reagents and resources. Therefore, a method that does not overload the qRT PCR with array of samples is desirable. This will also negatively impact the throughput of screening. The process described here (Fig. 3) will help in identifying uninfected sample pool and thereby the requirement of qRT-PCR-based high-value and efficient systems could be employed to the focused samples as a confirmatory test. The method will also help

in driving a super-high-throughput assay to score community transmission with a statistically validated model [5] for heavy density population, such as slum-dweller in Mumbai or migrant workers.

We hypothesize that the abundance of the mRNAs of the genes listed in Table 1 will be high at the early days of infection, and therefore, nucleic acid-based detection system will be more efficient. On the other hand, IgG- or IgM-based immunological principles may miss the window of the early detection as the titer of these proteins will reach the detectable limit in the blood after 4–10 days of infection (asymptomatic stage). Therefore, we have focused on developing process and designed probes and primers for the early detection of infection (1–4 days). In the Table 1, we have also listed probes which could be used for *in situ* hybridization assay [6]. The stringency of the forward and reverse oligonucleotides/PCR primers were checked *in silico* for potential secondary structure formation and primer dimerization using various *in silico* PCR tools available online. In the pipeline, we have added capillary electrophoresis to reduce time lag and increasing throughput of analysis. However, in the absence of such high-throughput capillary system agarose gel electrophoresis could also be used and gel loading with multichannel pipettes will be of help. Although, such manual intervention will reduce throughput but pooled sample analysis described in Figure 3 will still facilitate large-scale sample analysis. Kits and methods which work best for the proposed pipeline are derived by talking to more than 100 scientists working across different labs of India. However, kits with similar specifications could also be used based on the availability at different countries.

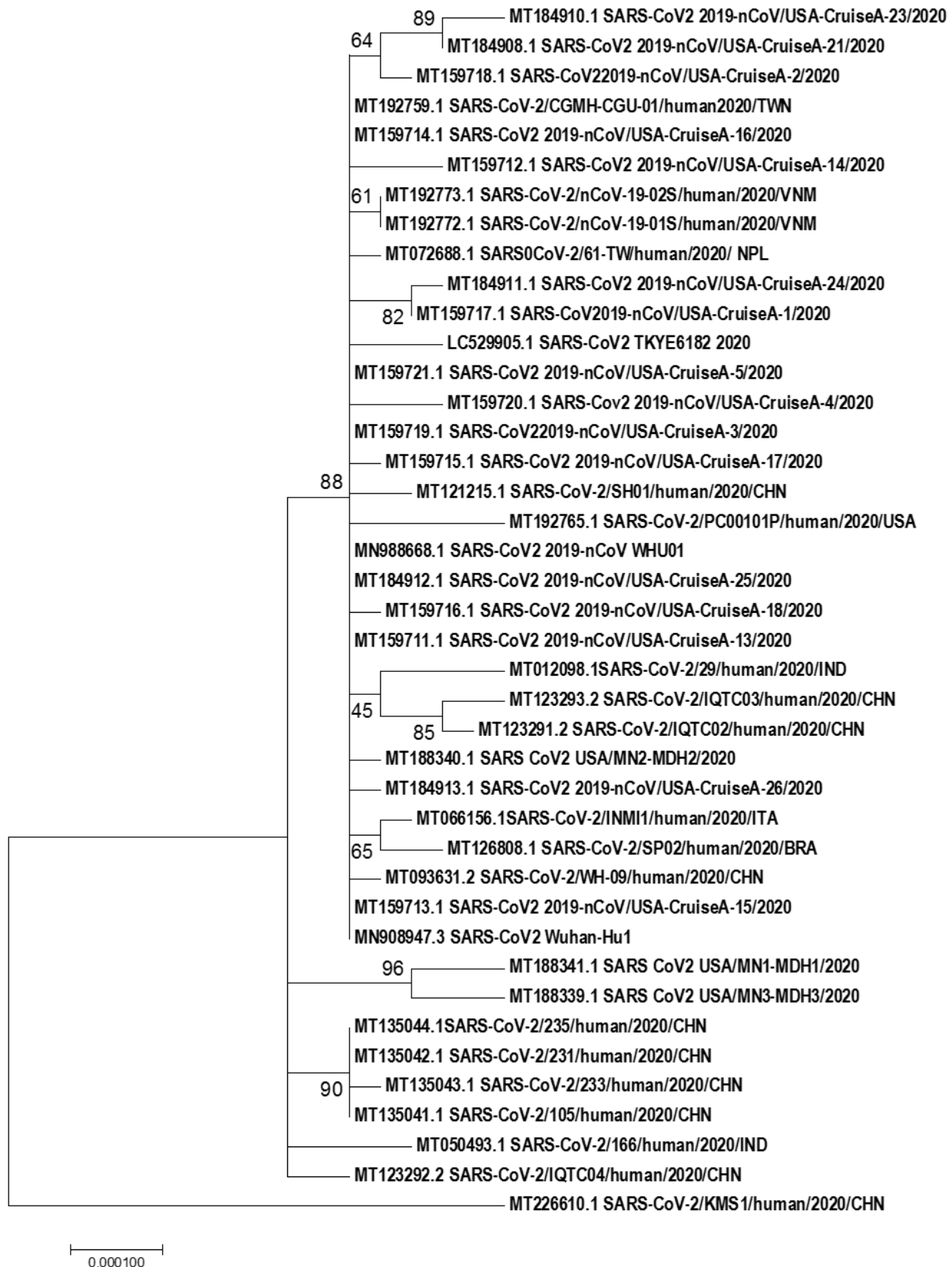


Figure 2: Neighbor-joining phylogenetic tree derived using 41 genome of SARS-CoV2 showing very high similarity between genomes of the virus sequenced in different countries. The tree was constructed with MEGA 7.0 software package and used the bootstrap value for 500 replications. Genome of MT226610 SARS-CoV-2/ KMS1/CHN was used to root the tree. The bar represents 1 base changes in 10,000 nucleotides.

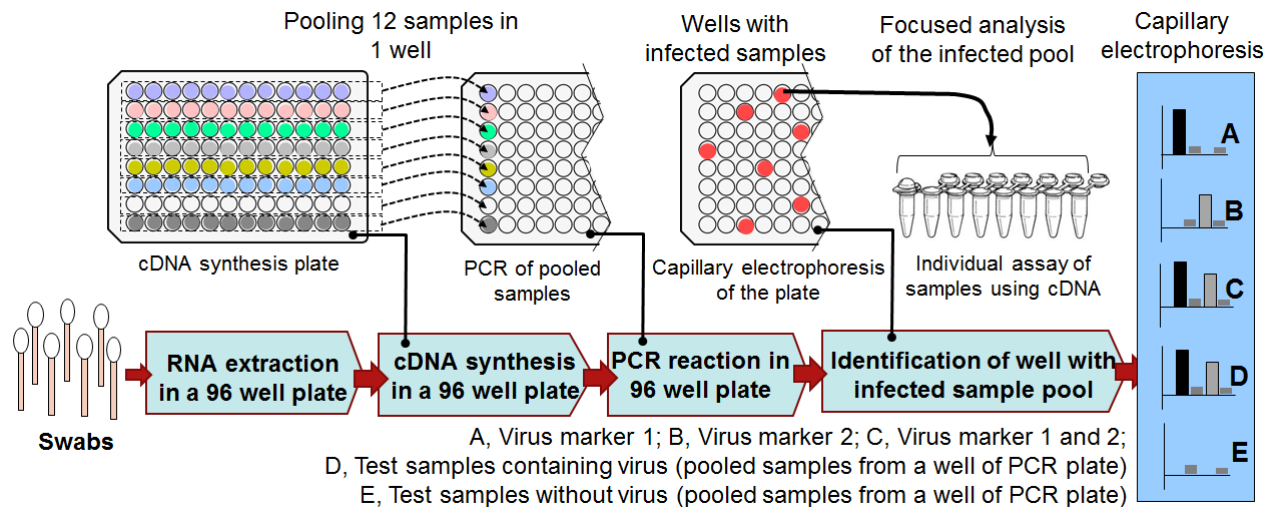


Figure 3: Schematic representation of super-high-throughput screening for COVID 19 infection using pooled-sample analysis. The method proposed here enables 1,000 samples analysis in one shot for stage-3 or community infection scoring.

2. CONCLUSION

Overall, the method of screening is not only efficient but also could be optimized to achieve high- to super-high-throughput sample analysis. This method will also help in decentralization of sample testing with minimal or no modification of existing infrastructure in universities, institutes or diagnostic centers across the country. We believe that our proposed pipeline of PCR-based sample screening will reduce dependency of qRT PCR machines and reagents and thereby, will enable large-scale community screening to combat the spread of COVID 19 by restricting it to stage 2 of the epidemic.

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CONFLICT OF INTEREST

The authors declare that they do not have any conflicts of interest.

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