

**REAL TIME RT-PCR ASSAY TO DETECT SARS-COV-2 IN THAILAND**

*Sutchana Tabprasit, Krongkan Saipin, Kamonwan Siriwatthanakul, Min Kramyoo, Watcharee Yokanit, Wuttikon Rodkvamtook, Kunakorn Kana, Pramote Imwattana, Thanainit Chotanaphuti*

**The Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand**

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**Abstract**

The Armed Forces Research Institute of Medical Sciences (AFRIMS) conducts medical research and disease surveillance to develop and evaluate medical products, vaccines and diagnostics to protect Royal Thai Army personnel from infectious diseases. Currently regarding globalized travel, infectious diseases pose a constantly evolving threat, indiscriminately transcending national, regional and even intercontinental boundaries. Since the COVID-19 outbreak, AFRIMS has gained knowledge from diagnostic tests for SARS-CoV-2 using the Centers for Disease Control and Prevention (CDC) as a reference protocol. We set up and developed the molecular diagnosis detection for SARS-CoV-2, Real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR), to analyze the nucleocapsid (N) genome of SARS-CoV-2 which is a standardized method in the laboratory. AFRIMS is certified by the Department of Medical Science, Ministry of Public Health (MOPH) as a COVID-19 laboratory network. We provided COVID-19 screening services to government units and private hospitals in late February 2020. It could be stated that AFRIMS is the first military unit to be certified by the MOPH. Since the COVID-19 pandemic started in Bangkok, 3,172 samples have been tested and 96 samples have been confirmed. Detecting viral RNA not only aids in the diagnosis of illness but also provides epidemiological and surveillance information.

**Keywords:** Real time RT-PCR, COVID-19, SARS-CoV-2

J Southeast Asian Med Res 2021: 5 (1): 21-25

<http://www.jseamed.org>

Correspondence to: Tabprasit S, Chief of Microbiology Section, Armed Forces Research Institute of Medical Sciences, Bangkok, Thailand

E-mail: [Sutchanat@afirms.org](mailto:Sutchanat@afirms.org), [Suttab@yahoo.com](mailto:Suttab@yahoo.com)

Received: 7 October 2020

Revised: 23 November 2020

Accepted: 03 December 2020

## Introduction

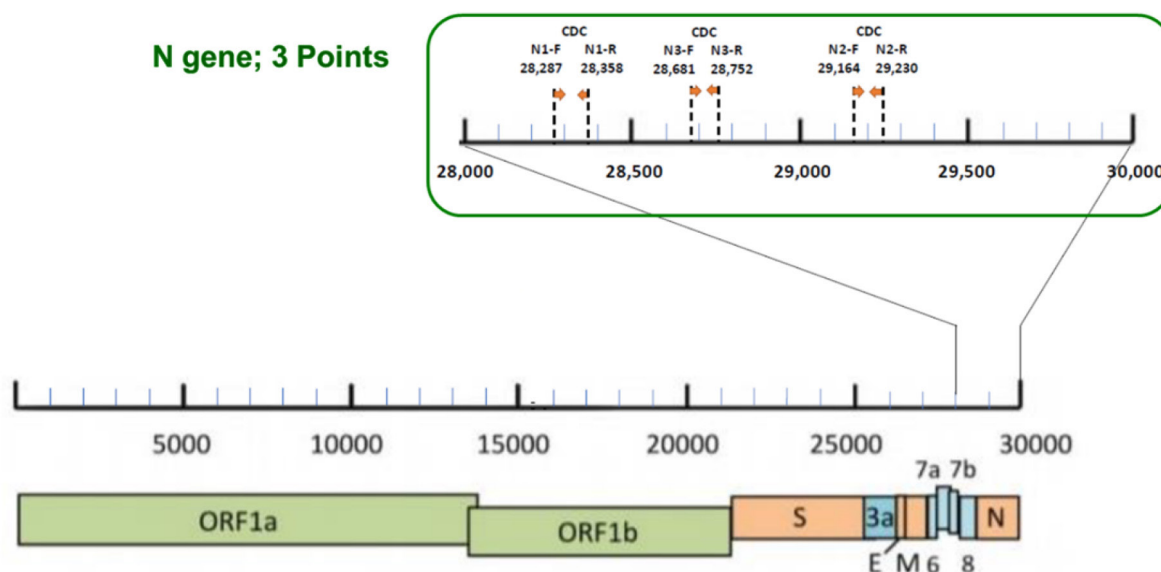
Emerging and reemerging pathogens constitute global problems for public health.<sup>(1)</sup> Coronaviruses are enveloped RNA viruses that cause diseases broadly among humans and other mammals such as respiratory, enteric, hepatic and neurologic diseases.<sup>(2,3)</sup> Six coronavirus species are known to cause human disease.<sup>(4)</sup> Four viruses, 229E, OC43, NL63 and HKU1, are prevalent and typically cause common cold symptoms among immunocompetent individuals.<sup>(4)</sup> The two other strains cause severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV). They are zoonotic in origin and during 2002 and 2003; SARS-CoV was the causal agent of severe acute respiratory syndrome outbreaks in Guangdong Province, China.<sup>(5-8)</sup> Later, MERS-CoV was the pathogen responsible for severe respiratory disease outbreaks in 2012 in the Middle East.<sup>(9)</sup> In 2020, the emergence of Coronavirus disease (COVID-19) outbreak, caused by a novel coronavirus, Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has spread globally. The outbreak of pneumonia caused by the SARS-CoV-2 continues to pose a serious threat to people's health. A better understanding of this new virus and developing ways to control its spread are needed imminently. However early detection and treatment is necessary for to prevent and control the outbreak. The SARS-CoV-2 was identified by RT-PCR<sup>(10)</sup> Specimens of the upper and lower respiratory tracts, such as bronchi or alveolar lavage and deep cough sputum were collected from each case, as well as serum from the onset and 14 days after onset.<sup>(11)</sup> At that time, testing in Thailand was limited to laboratories certified under the Department of Medical Sciences, MOPH so we attempted to set up Real time RT-PCR to diagnostically detect SARS-CoV-2 (COVID-19) to cope with outbreaks.

## Methods

We have used Real time RT-PCR assays for in vitro qualitative detection of SARS-CoV-2 in respiratory specimens.<sup>(12)</sup> (**Table 1**) A variety of RNA gene targets are used by different protocols, with most tests targeting 1 or more of the envelope (*env*), nucleocapsid (*N*), spike (*S*), RNA polymerase and *ORF1* genes.<sup>(12)</sup> The primer and probe sets are designed to specifically detect SARS-CoV-2. This protocol is used to detect three points of the N gene of SARS-CoV-2 (**Figure 1**). In addition, respiratory specimens include nasopharyngeal or oropharyngeal aspirates or washes, nasopharyngeal or oropharyngeal swabs, bronchoalveolar lavage, tracheal aspirates and sputum. Moreover, swab specimens should be collected only on swabs with a synthetic tip (such as polyester or Dacron®) with aluminum or plastic shafts. Swabs with calcium alginate or cotton tips with wooden shafts are unacceptable. Performing Real time RT-PCR amplification-based assays depends on the amount and quality of sample template RNA. RNA extraction procedures should be qualified and validated for recovery and purity before testing specimens. Commercially available extraction procedures have been shown to generate highly purified RNA when following manufacturer's recommended procedures for sample extraction. Retained residual specimens and nucleic extracts should be stored immediately at -70°C. We did not freeze/thaw extracts and specimens more than once before testing. Due to the sensitivity of Real time RT-PCR, these assays should be conducted using strict quality control and quality assurance procedures. A false negative result may occur when inadequate numbers of organisms are present in the specimen due to improper collection, transport or handling. The cycle threshold (Ct) is the number of replication cycles required to produce a fluorescent signal, with lower Ct values representing higher viral RNA loads. A Ct value less than 40 is clinically reported as PCR positive. The interpreted results are described in **Table 2**.

**Table 1.** Real time RT-PCR protocol  
Protocol preparation for real time RT-PCR

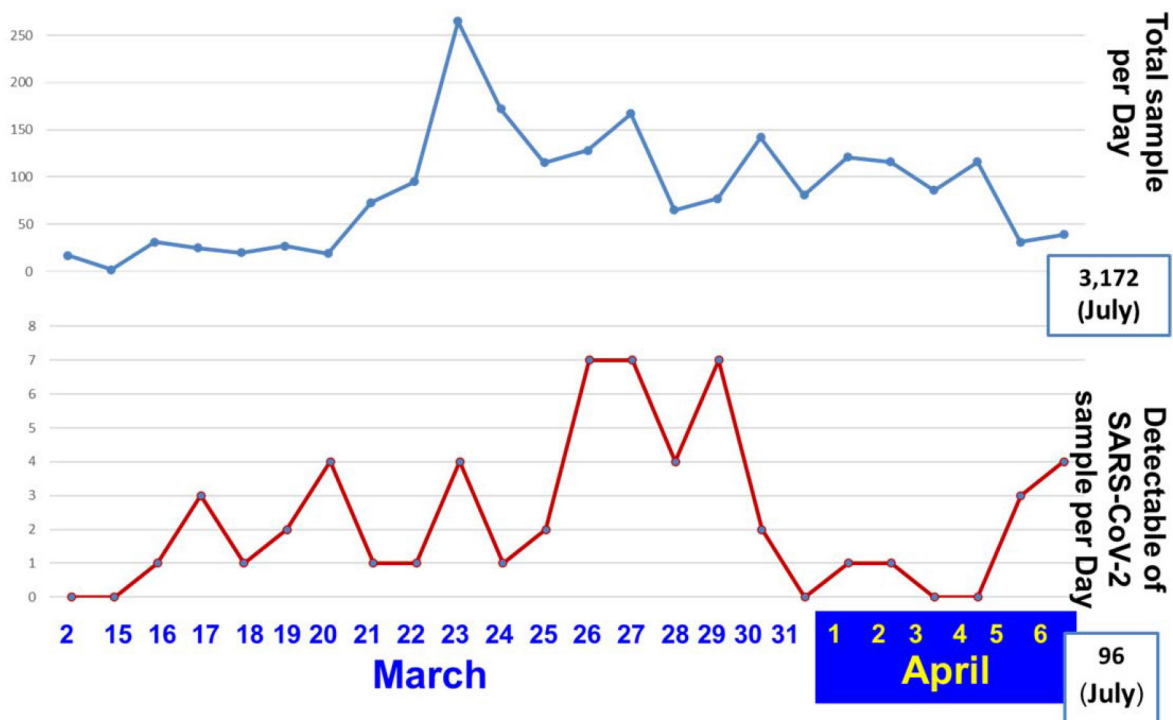
Step	Cycles	Temp	Time
RT incubation	1	95°C	3 min
Enzyme activation	1	50°C	30 sec
Amplification	45	95°C	10 sec
		55°C	30 sec



**Figure 1.** Detecting three points of the N gene of SARS-CoV-2 (N1, N2 and N3)

**Table 2.** SARS-CoV-2 real time RT-PCR diagnosis panel result interpretation

2019 nCOV_N1	2019 nCOV_N2	2019 nCOV_N3	RP	Result Interpretation <sup>a</sup>
+	+	+	±	2019-nCOV detected
If only one, or two, of three targets is positive			±	Inconclusive Result
-	-	-	+	2019-nCOV not detected
-	-	-	-	Invalid Result



**Figure 2.** Number of total samples and detectable SARS-CoV-2 samples daily

## Results

Timely and accurate laboratory testing of cases under investigation is an essential part of managing COVID-19 outbreaks. We should set up reliable SARS-CoV-2 testing laboratories to perform primary detection or confirmatory testing. AFRIMS is currently working closely with researchers so the diagnostics test will be set and validated promptly. As sequence information from the SARS-CoV-2 has recently been made available, PCR assays can be designed to detect these sequences. Moreover, sequence data can provide valuable information to understand the origin of a virus and how it spreads. After publishing the sequence of SARS-CoV-2, we set and validated the protocol to diagnostically detect SARS-CoV-2 (COVID-19). In February, all reagents, specific primers and specific probes were ready to perform Real time RT-PCR for COVID-19. After that, the virology laboratory was certified by the Department of Medical Sciences, MOPH 9 March 2020. However, Phramongkutklao Hospital sent the first specimen to AFRIMS in late February. In the middle of March, private hospitals sent COVID-19 suspected cases to diagnostically detect SARS-CoV-2.

Patients meeting the case definition for suspected SARS-CoV-2 infection should be screened for the virus using Real time RT-PCR. Since the COVID-19 outbreak, we obtained specimens to detect more than 3000 SARS-CoV-2 cases. Most cases came from the PMK Hospital and private hospitals. Ninety-six samples (3%) had the SARS-CoV-2 *N* genome, while 3,076 samples (97%) did not reveal the SARS-CoV-2 *N* genome as shown in **Figure 2**. In the beginning, detectable cases of SARS-CoV-2 or inconclusive results with external assistance were confirmed by the reference laboratory (Department of Medical Sciences, MOPH) that deployed the additional or confirmatory assays. To report detectable SARS-CoV-2 *N* genome results, all laboratories should follow the national reporting requirements, but in general, suspected cases should be reported to relevant public health authorities as soon as the laboratory receives a specimen. All test results, whether positive or negative, should likewise be immediately reported to authorities. Laboratories should also periodically report the number of test results to the MOPH weekly. The first wave of the COVID-19 outbreak in Thailand occurred in

the middle of March. At that time, we performed Real time RT-PCR to detect SARS-CoV-2 more than fifty samples daily. The highest number COVID-19 suspected cases totaled 262 samples from PMK and private hospitals 23 March 2020. COVID-19 infection has slightly decreased since April.

### Discussion

All users, analysts and any individuals reporting diagnostic results should be trained to perform this procedure by a competent instructor. They should demonstrate their ability to perform the test and interpret the results before performing the assay independently. Moreover, collecting multiple specimens (types and time points) from the same patient may be necessary to detect the virus. A false-negative result may occur when a specimen is improperly collected, transported or handled and may also occur when amplification inhibitors are present in the specimen or when inadequate numbers of organisms are present in the specimen. If the virus mutates in the Real Time RT-PCR target region, SARS-CoV-2 may be undetected or may be detected less predictably.

In every individual with COVID-19 infection, the Real time RT-PCR showed three point of the *N* gene and the PCR positive cases exhibited the same results with the reference laboratory (Department of Medical Sciences, MOPH). The number of suspected cases slightly increased in midMarch which was the same time as the first wave outbreak of SARS-CoV-2 in Thailand. Thus, we can use these principles as the model to detect unknown diseases in the future.

### Acknowledgements

The following people contributed to diagnostically detecting SARS-CoV-2 (COVID-19) using real time RT-PCR: Krongkan Saipin, Kamonwan Siriwatthanakul, Min Kramyoo, Watcharee Yokanit, Wuttikon Rodkvamtook, Kunakorn Kana, Pramote Imwattana and Thanainit Chotanaphuti.

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