



# RNA Internal Control (IC) for Routine Clinical Diagnostic Real-Time Reverse Transcription-PCR SARS-CoV-2 (RNA Internal Control for Routine rRT-PCR of SARS-CoV-2)

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**Abstract.** Since the beginning of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that source of a disease (COVID-19) pandemic in Indonesia, laboratories have to applied nucleic acid amplification tests (NAATs), namely real time Reverse-Transcriptase Polymerase Chain Reaction (rRT-PCR) as clinical diagnostic test. This method is extremely high sensitivity and speeds to diagnosis of virus infections. In order to obtain of appropriate in rRT-PCR's result, internal controls (ICs) has to issued assurance that clinical specimens are successfully amplified and detected. IC is allowing to provides the control being detected only if the target virus is absent and amplification is going well. IC can distinguish extraction failures, rRT-PCR restraint and technical errors relating to each individual sample. IC should be added at the samples prior to extraction. However, some commercial kit for rRT-PCR have available, but their performance for rules to add IC in within RT-PCR procedure has not yet been unassisted evaluated. The objective of this work was to estimated basic analytical of IC preparation for regular diagnostics of COVID-19. We were observed additional IC in RNA extraction and directly into the rRT-PCR reaction. In this study, we were used two commercial kit with conventional RNA extraction method (TIANamp Hi-DNA/RNA Kit) and automated viral RNA isolation (MagMAX™-96 Viral RNA Isolation Kit). The cycle threshold (Ct) were observed in treatment with additional IC in RNA extraction process, both of conventional RNA extraction method and automated RNA extraction method. Conversely, Ct were not observed in additional IC directly in rRT-PCR reaction. We conclude that IC should be added into sample in RNA extraction process.

**Keywords:** RNA · internal control · SARS-CoV-2 · Clinical diagnosis · Real time polymerase chain reaction

## 1 Introduction

The appearance of Severe acute respiratory syndrome (SARS) strains coronavirus 2 (SARS-CoV-2) have been described to infect humans and emerged in late 2020 in Indonesia and worldwide as coronavirus disease 2019 (COVID-19) [1]. This infection is associated with severe respiratory tract infections with broad range of symptoms, from asymptomatic to fever, dry cough, tiredness, aches, pains, multiorgan failure and in some cases, death [2]. SARS-CoV-2 has arrangements for transmitting from human to animals, conversely, by inhalation of infectious aerosols.

SARS-CoV-2 belonged to *Coronaviridae* family and Coronavirus genus. This virion has structure similar with SARS-CoV. The SARS-CoV-2 particle had a spherical morphology (the size of virus ~80 nm) and consists of a positive sense RNA (ssRNA+) with ~30 kb genome size, spike (S) glycoprotein which are protrude from the surface of virions, membrane (M) glycoprotein, nucleocapsid (N) protein which are protein that forms associated with genomic RNA, envelope (E) and an additional glycoprotein with acetyl esterase and hemagglutination (HE) properties [3, 4].

Laboratory testing and diagnostics of SARS-CoV-2 is key role for the analytic and therapy management of patient and the execution of disease control at population level. Until now, the gold standard diagnostic examination for SARS-CoV-2 inflammation is viral RNA diagnosis by reverse transcription real-time Reverse-Transcriptase Polymerase Chain Reaction (rRT-PCR). This method has high sensitivity, specificity and accuracy for SARS-CoV-2 detection [5]. Real time RT-PCR (rRT-PCR) assays use two or three targets of SARS-CoV-2 genes, namely E gene (5'-ACACTAGCCATCCTTACTGCGCTTCG-3'), RdRP (RNA-dependent RNA polymerase) gene (5'-AGATGTCTTGTGCTGCCGGTA-3'), and N (Nucleocapsid) gene (5'-TGGGGYTTTACRGGTAACT-3'). The specimen for rRT-PCR is collected from nasopharyngeal (NP) swabs, oropharyngeal (OP) swabs, sputum, tracheal aspirates, bronchoalveolar lavage and pleural fluid. This specimen should be taken from the primary targets of the SARS-CoV-2 disease [6].

The general workflow for nucleic acid examination for SARS-CoV-2 is consist of three steps, namely RNA extraction, reverse transcription process and amplification of gene by RT-PCR assays. Performance viral RNA isolation is a crucial for execution of rRT-PCR assays [7]. In order to obtained reliable result of rRT-PCR analysis, Internal Controls (ICs) needs to be added during nucleic acid testing workflow [8]. IC is providing assurance that viral RNA has successfully extracted from specimen and amplified. A positive signal of IC indicated the amplification of gene target was sufficient to revealed a positive result [9].

Further, some Virus RNA Extraction Commercial Kit and SARS-CoV-2 Test Kit has provided for routine diagnostic. However, when the IC should be added during nucleic acid testing workflow was not clear. Some test kits provide information to added the IC in the RNA extraction process and some test kits added before rRT-PCR assays. Therefore, analysis that provides information when the IC should be added during testing is urgently required [9, 10].

Here, we present a study to compare basic analytical of IC preparation for routine diagnostics of SARS-CoV-2. We determined whether IC should be added during RNA

extraction or directly into rRT-PCR assays. Our result can be embraced into supplement for molecular diagnostic examination for COVID-19.

## 2 Methods

### 2.1 Samples and Internal Controls Used for RRT-PCR

For biosafety purposes, samples were Viral Transport Medium (VTM) which added Positive Control (PC) plasmids from commercial kits, namely Allplex™ 2019-nCoV Assay (Cat no. RP10252W). This plasmid containing E gene, RdRp gene and N gene. IC was obtained from Allplex™ 2019-nCoV Assay.

### 2.2 Total RNA Extraction

Total RNA isolation was carried out in line with the manufacturer's information, 2 kits, namely the TIANamp Hi-DNA/RNA Kit (Cat. No. DP315-T8) and the MagMAX™-96 Viral RNA Isolation Kit (Applied Biosystems, Cat. No. AMB1836-5).

The first TIANamp Hi-DNA/RNA Kit were performed manually as follows: As much as 50 µL of VTM (with PC plasmid and 10 µL of IC) were added by 20 µL of Proteinase K of concentration 20 mg/mL and 600 µL of RLC Buffer (containing guanidiniumhydrochloride). Suspension were homogenized and incubated 10 min at 56 °C until the cell was totally dissolved. A 600 µL of isopropanol were added to the microcentrifuge, and vortexed for 2 min. Liquid in the centrifuge microcentrifuge were transferred to a RNase-free Spin Column CA4 and rotate at very high speed for 1 min on centrifuge at 12,000 rpm in 4 °C (~13,400 × g). Some fluid flows through were wasted in the Spin Column CA4. A 500 µL of GD Buffer (after absolute ethanol added) were added to the Spin Column CA4 and centrifuged for 1 min on centrifuge at 12,000 rpm in 4 °C (~13,400 × g). Some fluid flows through were discarded. Furthermore, a 500 µL of RW Buffer were added and centrifuged for 1 min on centrifuge at 12,000 rpm in 4 °C (~13,400 × g). Spin Column CA4 were placed in a new 1.5 mL of RNase-Free microcentrifuge and added by 50 µL of RNase-free ddH<sub>2</sub>O to the middle of the adsorption membrane. Microcentrifuge were centrifugated for 1 min on centrifuge at 12,000 rpm in 4 °C (~13,400 × g) and collected the samples.

The second kits, MagMAX™-96 Viral RNA Isolation Kit were performed using The MagMAX™ Express (ThermoFisher Scientific) Machine as follow: As much as 50 µL of VTM (with PC plasmid and 10 µL of IC) were put in to the appropriate wells of the Processing Plate. Processing Plate were shaken for 1 min on an orbital shaker at highest rate. A 130 µL of Lysis/Binding Solution were added and shake the Processing Plate for 5 min on an orbital shaker at the maximal speed. Processing Plate were moved to a magnetic stand to detain the Binding Beads Virus RNA. The plate was leaved on the magnetic stand for not less than 3 min. If detention is appropriate, the Binding Beads

Virus RNA appearance pellets opposed to the magnets in the magnetic stand. Supernatant was rigorously aspirated and the Processing Plate was removed from the magnetic stand. A 150  $\mu\text{L}$  of Wash Solution 1 (make sure isopropanol added) and Wash Solution 2 (make sure ethanol added) were applied, respectively. Processing Plates were shaken without a lid for 2 min to dry the beads. A 90  $\mu\text{L}$  of Elution Buffer was added and supernatant was transferred to a nuclease-free microcentrifuge.

### 2.3 RRT-PCR Conditions

In this assay, we prepared four features of microcentrifuge, namely microcentrifuge I: IC control added directly to the rRT-PCR reaction; microcentrifuge II: sample from TIANamp Hi-DNA/RNA Kit added with IC; microcentrifuge III: sample from Allplex™ 2019-nCoV Assay added with IC; microcentrifuge IV: negative control (rRT-PCR reaction without RNA templates).

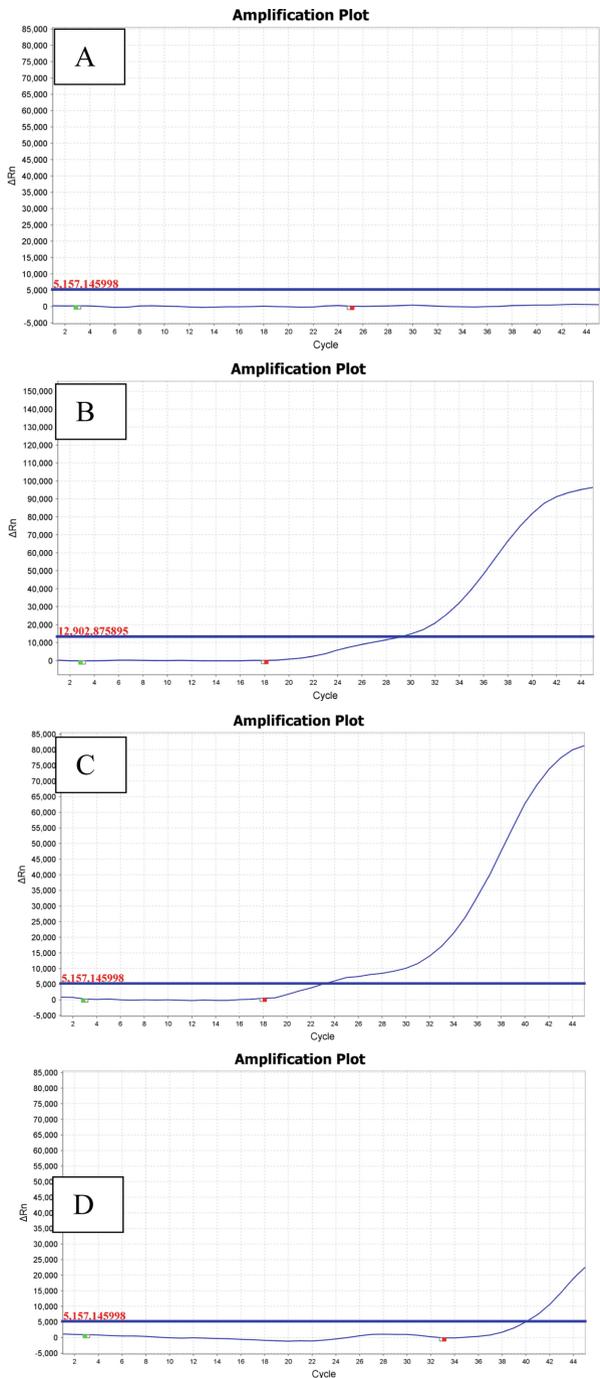
In this study, rRT-PCR for COVID-19 were used Allplex™ 2019-nCoV commercial kits assay from Seegene Manufacturer (Seoul, South Korea) and QuantStudio™ 5 Real-Time PCR (ThermoFisher Scientific). Nucleic acid was detected by genes encoding for envelope protein of SARS-CoV-2, RdRP gene of SARS-CoV-2, N gene of SARS-CoV-2 and the internal control (IC) which are indicated by Traditional fluorescein (FAM), CAL Fluor Red 610 fluoresces, Quasar® 670 and HEX™ channels, sequentially. Each reaction consists of eight  $\mu\text{L}$  of standard RNA synthesis or RNA extraction products were mixed to 17  $\mu\text{L}$  of the final volume reaction mixture. This mixture consists of 2  $\mu\text{L}$  of Real-time One-step enzyme, 5  $\mu\text{L}$  of 2019-nCoV Master Mix (probe and primer), 5  $\mu\text{L}$  of Real-time One-step Buffer (5X), and 5  $\mu\text{L}$  of RNase-free Water. rRT-PCR condition was reverse transcription step involved incubation in 1 cycle at 50 °C for 20 min, then followed by polymerase chain reaction. Thermocycler temperature was increased for pre-95 °C in 15 min continued 45 cycles at denaturation process (at 95 °C for 15 s), thus annealing temperature at 58 °C for 30 s. In addition, the samples were conquered to melting curve analyses (5 s denaturation at 95 °C and followed by at 65 °C for 1 min accompanied with a gradually raise in temperature up to 97 °C with constant monitored of fluorescence) to determine the precision of the examination.

The test outcome was defined as results from the Ct or a cycle threshold (Ct) value of the recommendation gene and reported by descriptive. Sample is positive detected (valid) when the Ct value of the Real Time RT-PCR Test recommendation gene was  $\leq 22$  and not detected (invalid) when  $> 22$  (Seegene).

## 3 Results

We performed rRT-PCR using samples from two commercial kits, microcentrifuge reaction with IC which are added directly before rRT-PCR, and negative control to determine the crucial of IC in nucleic acid testing workflow. To this end, Ct values were not measured in the reaction in the nonappearance of IC in RNA extraction process (Fig. 1).

Toward to Ct value (Table 1), we found that the Ct values were detected in all of sample reaction by using commercial kit which added by IC before RNA extraction process.



**Fig. 1.** Internal Control (IC) amplification plot in Real-time polymerase chain reaction (rRT-PCR). The result represents the linier plot result for (A) negative control of IC, (B) IC control was added before RNA extraction in TIANamp Hi-DNA/RNA with IC, (C) IC were directly added into rRT-PCR reaction in MagMAX -96 Viral RNA Isolation Kit with IC, (D) IC added directly in rRT-PCR

**Table 1.** Comparison of IC Application during Nucleic Acid Testing Workflow.

Samples	Treatment		
	TIANamp Hi-DNA/RNA with IC	MagMAX -96 Viral RNA Isolation Kit with IC	IC added directly in rRT-PCR
Result	Valid	Valid	invalid

a. test was run duplicate

## 4 Discussion

rRT-PCR has been applied in SARS-CoV-2 in clinical diagnostic tools. This method is providing rapid, specific and sensitive detection of RNA viral in a variety of samples [9]. In some case, false-positive of false-negative result could be generated during workflow (10). It will be caused by reaction contamination or unappropriated reagent. In order to avoid this false result and minimalize error of testing workflow, either RNA extraction or rRT-PCR reaction, some control should be added, namely internal control (IC), negative and positive control [11].

An internal control (IC) is crucial to determined efficiency RNA extraction from sample and as prevent a misfiring in some step of the nucleic acid analysis. A positive IC result confirms that the amplification has succeeded. In addition, a negative rRT-PCR test result is truly result [6, 11–13].

This study demonstrated that IC is a part of nucleic acid evaluate flow of work for diagnosis of SARS-CoV-2. It should be noted that IC is RNA contain gene target of SARS-CoV-2 and reliable for virus diagnosis. IC should be added to samples together in extraction process. In this case, the volume of IC is added to all specimen in same volume. Therefore, the Ct can be detected at the same value in all sample.

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